PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

SUNDAY, 3/4/16

media preparation for yeast culture and protoplast formation

Optimized protocol for Spheroblasts and PEG uptake

Introduction

Determined with blood, sweat & tears

Materials

-
  - YPD; yeast
  - LB; E. coli (or other endosymbiont in corresponding medium)
  - Softening medium
  - Spheroblasting medium
  - Zymolase
  - PEG (~20000)
  - 0.7M Sorbitol in YPD, pH 5.4

Procedure

1. Grow cells in liquid culture to mid-logarithmic stage (oD 0.5 - 0.6), aliquot cells to 50ml falcons
2. Centrifuge the cells at 5000 x g for 5 min
3. Resuspend the cell pellet in sterile water at a concentration of oD 10 (=2.5ml)
4. Centrifuge at 5000 x g for 5 min at RT
5. Resuspend the cell pellet in “softening medium” at a concentration of oD 10 (=2.5ml)
   softening medium: 100 mM Hepes-KOH, pH 9.4 (1 M stock, RT)
   10 mM dithiothreitol (DTT) (1 M stock, -20˚C)
   *always prepare fresh softening medium
   *Hepes-KOH may be substituted with another buffer (e.g., Tris pH 9.4 or Pipes-KOH pH 9.4)
6. Incubate for 15 min at RT
7. Centrifuge at 5000 x g for 5 min at RT
8. Resuspend cell pellet in “spheroplasting medium” at a concentration of oD 5 (=5ml)
   spheroplasting medium: 1X YNB (10X stock, RT)
   2% glucose (50% stock, RT)
   1X amino acids (100X stock, RT)
   50 mM Hepes-KOH, pH 7.2 (1 M stock, RT)
   1 M sorbitol (2 M stock, RT)
   *spheroplasting medium can be stored at RT indefinitely
9. Add 250U of Zymolase (= 10µl of an 1:1 dilution of 500U Zymolase with water)
   *stock = 10 mg/ml in 1X PBS, 1 M sorbitol (store in aliquots at -80˚C)

10. Incubate for 60 min at 30°C (or an otherwise appropriate temperature; for instance, if the strain has a temperature-sensitive growth defect, incubate at the permissive temperature)
11. Centrifuge at 3000 x g for 3-4 min
12. Resuspend spheroplasts in spheroplasting medium at concentration of oD 10 (=2.5ml)
13. Prepare samples containing 500µl with your PEG of choice
14. Add 500µl of the yeast spheroblasts to each sample
15. OPTIONAL: add a small amount (~50µl) of the regeneration medium (0.7M Sorbitol)
16. Add 500µl E. coli at a concentration of oD 1-2 (dilution might be necessary)
17. Let the mix incubate for 20' at 30°C and 110rpm
18. After inverting, centrifuge mix for 15' with 1000 x g
19. Discard supernatant and resuspend to 1.5ml with 0.7M Sorbitol in YPD (pH 5.4). There will probably be remaining PEG in the tube, be sure to resuspend this too.
20. Regeneration of the cells for ~2h in 30°C with 110rpm

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TUESDAY, 5/4/16

Table1

	A	B	C	D	E	F
1	**YPD (1L)**	**YPD +2% Agar (400ml)**	**Protoplast buffer 2 (50ml)**	**Protoplast buffer 1/3 (50ml), pH 8.0**	**Protoplast buffer 4 (50ml), pH 5.8**	**1M DTT Stock solution, 8ml**
2	10g Yeast extract	4g Yeast extract	9g Sorbitol	9g Sorbitol	9g Sorbitol	1,23g DTT in water, steril filtrated
3	20g Peptone	8g Peptone		0,38g EDTA	0,015g EDTA	
4	20g Glucose	8g Glucose			0,145g tri-sodium citrate di hydrate	
5		8g Agar Agar, danish				

Prepared the media as descirbed above. All Solutions were autoclaved, except for DTT Stock solution. DTT Stock solution was frozen in 500µl Aliquots. Will be added to Protoplast buffer 2 right before use.

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WEDNESDAY, 6/4/16

Inoculate 50ml YPD with SNK, incubate at 30°C until OD600=0.5-0.6.

Transformation of Methylobacterium extorquens WT:

Plasmids: PTE770; PTE767 (mCherry)
Medium: sterile NB Medium

50µl of Methylobacter cells, 1µl of a Plasmid and 1ml NB medium were mixed in 2ml Eppendorf tubes.
The cells were electroporated and incubated for 3-4h on 30°C.
After the incubation the cells were plated on Agar plates.

Perparation of Stock Solutions

Table2

	A	B	C	D
1	**1M Glucose (250ml)**	**1M Tris-HCl, pH 8 (100ml)**	**1M Tris-HCl, pH 7.5 (100ml)**	**0,25M EDTA, pH 8 (25ml)**
2	49,54g Glucose monohydrate	15,7g Tris-HCl	15,7g Tris-HCl	2,33g EDTA disodium-dihydrate

EDTA Stock solution was sterile filtrated, the rest was autoclaved.

Inoculate 50ml of SNK 76 (CEN PK1 13.71C) o/n on 25°C

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THURSDAY, 7/4/16

Measurement OD600 of SNK 76 o/n culture.
OD600 = 0.5 (10:00)

Start of Protoplast protocol
Centrifuge for 10mins, 1,500g
(see protocol)
used Zymolase concentration 0.2U/ml

Preparation of glycerolstocks of E.coli SNK12 (NEB Turbo)
Yeast SNK76 (CEN PK1 13.71)
directly from the plates
in 25% glycerol endconcentration. Freeze at -80°C.

Preparation of 5% SDS solution in dH2O for protoplast efficiency.
Protoplast efficency:
At 5, 10,20,30,40,50, and 60 minutes 200µl protoplasts are mixed with 2ml of sorbitol and 800µl 5% SDS solution and OD800 is measured immediately (Tab.3).
Protoplast efficiency= 100-(tx(OD800)/t0(OD800))*100

Table3

	A	B
1	Timepoint t	optical density (800nm)
2	0	0.367
3	5	0.337
4	10	0.282
5	20	0.245
6	30	0.214
7	40	0.194
8	50	0.175
9	60	0.157

Protoplast efficency after 60min ~58%
possible reasons for low efficency: Zymolase concentration too low for amount of used cells.

Microscopy of protoplast solution showed similar results: some protoplasts were formed but only few.

Trying it again tomorrow with higher zymolase concentration

- ☒ inoculate fresh yeast culture

- ☒ prepare 50% glycerol solution (TA?)
- ☒ 1M Sorbitol (500ml). autoclave.
- ☒ plate yeast from gly-stock

Inoculated 250ml YPD with CEN PK1, incubate o/n at 25°C. (Used for Zymolase assay /w different concentrations.)

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FRIDAY, 8/4/16

Harvested at OD600=1.0, next time use pre and mainculture to have fresh cells.

Protoplast efficiency:

Table4

	A	B	C	D	E	F	G
1	timepoint t	1U Zymolase	2U	4U	6U	8U	
2	0	0.63	0.655	0.679	0.555	0.493	
3	5	0.43	0.364	0.072	0.174	0.128	
4	10	0.388	0.38	0.242	0.105	0.064	
5	20	0.239	0.183	0.12	0.04	0.027	
6	30	0.151	0.113	0.068	0.027	0.01	
7	40	0.107	0.06	0.047	0.035	0.007	
8	50	0.07	0.02	0.028	0.014	0.002	
9	60	0.05	0.057	0.013	0.004	0.003	
10	PE after 60min [%]	92.0634920635	91.2977099237	98.0152671756	99.3893129771	99.5419847328	

For 1 Unit Zymolase at t=50 protoplast efficiency is already at 83%. Probably the working concentration of zymolase from yesterday was gone bad (too warm?). Higher zymolase concentrations probably killed all cells, we'll check under the microscope.

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SATURDAY, 9/4/16

Inoculated 150ml of YPD with SNK 76 on 30°C.
followed protocol. see table 5 for SDS Assay.

Table5

	A	B	C	D
1	timepoint t	1U/µl (dilution from friday)	1U/µl	0.5U
2	0	0.158	0.152	0.181
3	5	0.113	0.082	0.0134
4	10	0.073	0.045	0.092
5	20	0.035	0.022	0.064
6	30	0.025	0.01	0.037
7	40	0.017	0.008	0.017

after 60min incubation with zymolase, we prepared a calcoflour staining. to 1ml of PP suspension we added 4µl (3mg/ml) Calcoflour solution.
Fluorescence microscopy showed little to no protoplast formation for each concentration. highest PP efficency was approx. 5%.
Most of the cells still have a cell wall.

Prepared Hepes-KOH buffers (pH 9,4; pH 4,2) for new yeast protoplast protocol.
buffers were prepared in a total volume of 0,1 l with 23,8 g of Hepes. pH was set with 10 M KOH.
Buffers were then autoclaved.

Prepared spheroplasting media for new yeast protoplast protocol.
media was prepared in a total volume of 0,5 l.
- 0,3 g YNB/Aminoacids
- 2,25 g Glucose
- 0,025 l Hepes-KOH (pH 7,2) total concentration of 50 mM
- 91,1 g Sorbitol

prepared a preculture o/n of SNK76 on 30°C.

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MONDAY, 11/4/16

OD600 (1:10 dilution) of preculture=0.672
for inoculating 100ml of YPD on OD600=0.2 we used 3.0ml of pre culture
Inoculated 100ml main culture at OD600=0.218. (9:15)

Table6

	A	B	C	D
1	timepoint t	concentrated zymolase	1:2 diluted zymolase	
2	0	0.085	0.119	1:15
3	5	0.024	0.05	1:15
4	10	0.009	0.023	1:15
5	20	0.002	0.009	1:15
6	30	0.001	0.007	1:15
7	40	0.002	0.006	1:10
8	50	0.001	0.002	1:10
9	60			

Yeast cells were observed with fluorescence microscopy. Cell wall was marked with Calcoflour.
About 70% of the cells showed no blue fluorescence.

SNK76 o/n culture on 30°C.

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Project: iGEM 2016
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TUESDAY, 12/4/16

Inoculated 100ml of YPD with SNK76 (OD600=0.2). Let it grow on 30°C till OD600=0.5.

prepared the following solutions:

Table7

	A	B	C	D	E
1	0.1M MgCl2	0.1M CaCl2 + 14% (w/v) glycerol	stabilization bufffer (protoplasts) 100ml	50% (v/v) glycerol in water	
2	0,55g MgCl2-dihydrate	0,5g CaCl2-tertahydrate	2.08g KCl	25ml glycerol	
3		7ml glycerol	0.11g CaCl2	25ml ddH2O	
4			0.05g MgCl2		
5			0.06g Tris/MES		
6			1g Glucose		
7					
8			pH 7.2		

all buffers were sterile filtrated.

For protoplasts the culture was splitted into two.

1. 5µl Zymolase (2U/µl)
2. 10µl Zymolase (2U/µl)

Microscopy showed approx. 70% protoplast formation. staining was done with calcoflour.
Yeast protoplasts were stored in stabilization buffer o/n on 25°C.

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Project: iGEM 2016
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WEDNESDAY, 13/4/16

Preparation of 2% Agarslides for microscopy of the o/n protoplasts.
stained with calcoflour, all protoplasts were bursted. only few cells survived (but had a cellwall...)

inoculated 100ml of NEB Turbo in LB for competent cells.
competent cells were prepared and frozen in 200µl aliquots on -80°C.

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THURSDAY, 14/4/16

- ☒ preparation of M. extorquens media (except MeOH!)
- ☒ inoculating 1L SNK76 for cell lysis. starting OD600=0.5
- ☐ Monday: Cell Lysis via homogenizator @ ZSM /w Anne

  pos control: corresponding media
  neg control: tethering buffer (10 mM potassium phosphate [pH 7.0], 0.1 M NaCl, 100 µM EDTA, 10 µM l-methionine, 20 mM sodium l-lactate)

  1: E. coli in celllysate
  2: M. extorquens in celllysate
  3: B. subtilis (?) in celllysate

- ☒ Monday: Inoculating M. extorquens AM1, E.coli /w pFAB
- ☒ Ordering primers for knockout cassette
- ☒ 1M Hepes-KOH pH9.4 was contaminated, prepare a new one!

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Project: iGEM 2016
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FRIDAY, 15/4/16

Inoculated preculture SNK76 in YPD o/n 30°C.
Inoculated M. extorquens AM1 in MM on 30°C.
Inoculated E.coli in LB o/n on 37°C.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

MONDAY, 18/4/16

- ☒ Prepare 1M Hepes-KOH pH9.4
- ☒ getting tethering buffer from Anne
- ☒ plates LB & MM for Methylo
- ☒ Homogenizer.

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Project: iGEM 2016
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Dates: 2016-04-03 to 2016-10-11

TUESDAY, 19/4/16

Prepared 45%(w/v) PEG 3350 in water containing 75mM CaCl2.
Dissolved 0.42g CaCl2 dihydrate, 22.5g PEG 3350 in dH2O.

Prepared 100ml 1M Tris-HCl, dissolved 12,1g in dH2O, pH adjusted to 8.0 with HCl.

Need to prepare a 100ml 40% (w/v) sucrose solution, getting sucrose from Anne?

at 14:00: harvested SNK76 from friday in ultracentrifuge 6.000g for 30min.
homogenized cells in bead beater 10x for 10sec with 2min cooling in between each step.
lysate (supernatant) was pooled in 50ml falcons after centrifuging 5min at 6.000g (4°C).

lysate was centrifued again in ultracentrifuge (100.000g) for 1h to seperate cellwall/membrane from cytosolic part.

for growth tests 15ml falcons were prepared:

for each strain (e.coli, B.sub WT, M. extorquens AM1 767/770) 5ml as follows:

pos. con: medium /w corresponding antibiotics
neg. con: Tethering buffer
neg. con: PBS
celllysate

The different media were inoculated with 100µl of each strain.
OD600 was recorded

Table8

	A	B	C	D	E	F	G
1		E.coli, 30°C	B.subtilis	M. ex 770	M. ex 767	E.coli, 37°C	B. subtilis
2	neg con Tethering Buffer	0.062	0.023	0.01	0.012	0.06	
3	neg. con PBS	0.062	0.022	0.012	0.018	0.06	
4	pos con MEDIA	0.08	0.03	0.012	0.012	0.09	
5	yeast celllysate	0.627	0.601	0.425	0.558	0.506	

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Project: iGEM 2016
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Dates: 2016-04-03 to 2016-10-11

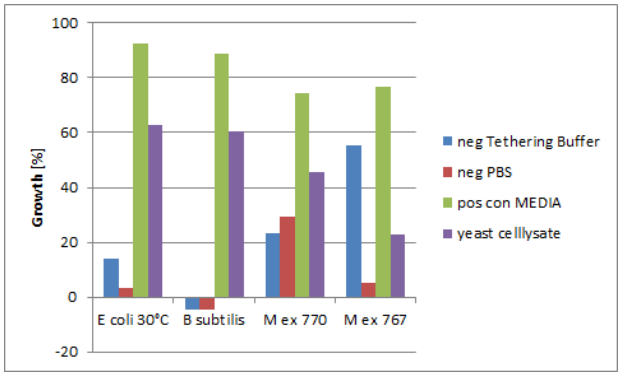
WEDNESDAY, 20/4/16

OD600 was measured after incubation o/n n 30/37°C

Table9

	A	B	C	D	E	F	G
1		E coli 30°C	B subtilis	M ex 770	M ex 767	E coli37°C	B subtilis
2	neg Tethering Buffer	0.072	0.022	0.013	0.027	0.074	
3	neg PBS	0.064	0.021	0.017	0.019	0.062	
4	pos con MEDIA	1.046	0.267	0.047	0.051	1.8	
5	yeast celllysate	1.684	1.514	0.779	0.723	1.578	

clipboard_2016-04-20_114130.png



(These data suggests roughly the growth of bacteria in cell lysate. It is no significant data.)

Each cell suspenson was observed by light microscopy. (Data can be found in Uploads under Yeast cell lysate 20.04.16)

100 µl of each cell suspension was plated on LB (B. subtilis), LB-Kan (E. coli), Minimal Media + Tet (M. ex 770/767). Plates were then stored at 30°C and 37°C (E. coli) o.n.

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THURSDAY, 21/4/16

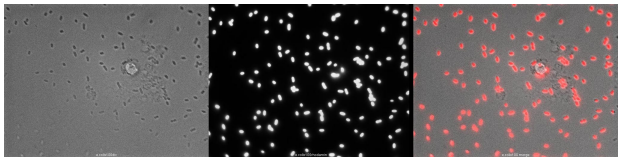
Inoculate 100 ml YPD with preculture (OD600= 0.25) for 4 hours.
Cells at OD600 of 0.5 were harvested and protoplasts were prepared by protoplasts protocol.

PEG induced uptake: 200µl of yeast protoplast & 800µl of o/n cultured bacteria were mixed, all other steps were done according to the protocol.

PEG induced uptake of Yeast Protoplasts

#believe.

e.colix100 montage.jpg



PEG fusion

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Dates: 2016-04-03 to 2016-10-11

FRIDAY, 22/4/16

Inoculate M. extorquens 770 and 767 (15ml) for incubation at 30°C over the weekend in MM + Methanol + Tetramycin
Inoculate 1L yeast in YPD for yeast lysate experiment

PEG fusion

Project: iGEM 2016
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Dates: 2016-04-03 to 2016-10-11

MONDAY, 25/4/16

Preparation of yeast celllysate.
Inoculation E.coli /B.subtilis/ M.extorquens 767/ M. extorquens 770 in

5ml each:
100% EtOH
PBS
Tethering Buffer
Yeast celllysate
LB+Kan/LB/MM+Tet/MM+Tet
OD600 0.2
o/n on 30°C.
for control 5ml of pure yeast celllysate was handled as the rest of the samples.

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TUESDAY, 26/4/16

OD600 of each sample from monday was measured.

Table11

	A	B	C	D	E
1		E. coli	B. subtilis	Methylobacterium 770	Methylobacterium 767
2	Tethering buffer	0.65	0.225	0.942	0.688
3	PBS	0.35	0.44	0.365	0.31
4	LB/Minimal Media	1.865	0.375	0.761	0.715
5	Yeast cell lysate	1.492	0.366	0.617	0.441
6	Ethanol	0.518	0.256	0.541	0.45

(conclusion: lysate seems to be fine for them!!!)

Each sample was used to gather data with FACS analysis.
(insert conclusion here!!!)

MG1655 was plated on LB agar plates and incubated over night to get rid of remaining plasmids.
Preculture of pFAB 3677 in 5ml LB + Kan for miniprep

Tryout for different PEG-solutions:
Protoplasts were prepared like before using a 70ml culture with an end volume of 7,2ml for oD 5
PEGs used were 3350, 4000 and 6000, each in a 45%(w/v) solution with 75mM CaCl2 for the standard protocol of PEG induced uptake of yeast

OD measurement of every cell culture for estimated cell number:

Table12

	A	B	C	D
1	Strain	oD (1:10)	oD net [ml]	
2	E. coli	0.583	5.83	oD 1 = 8*10^8 cells
3	B. subtilis	0.263	2.63	oD 0.5 =10^8 cells
4	M. extorquens 770	0.04	4.04	Same as coli due to lack of sources
5	M. extorquens 767	0.4	4	Same as coli due to lack of sources

For yeast: oD 1.04 = 2x10^7 cells

Two different bacterial cell concentrations were used, therefore stock solutions are necessary

Table13

	A	B	C
1		2x10^9 [Stock A]	2x10^7 [Stock B]
2	E. coli	1:1.9 [1.9ml LB and 1ml cells]	1:32 [2.906ml LB and 94µl cells]
3	B. subtilis	Not possible, 792µl were used	1:26 [2.885ml LB and 115µl cells]
4	M. extorquens 770	1:1.9 [1.9ml MM and 1ml cells]	1:32 [2.906ml MM and 94µl cells]
5	M. extorquens 767	1:1.9 [1.9ml MM and 1ml cells]	1:32 [2.906ml MM and 94µl cells]

Setup:

Table10

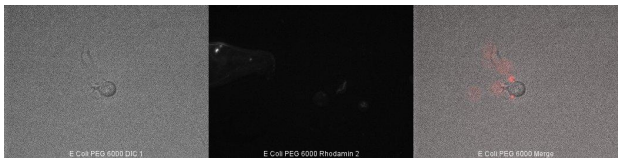
	A	B	C	D	E
1		Y [208µl] + 2x10^9 bacterial cells	Y [208µl] + 2x10^7 bacterial cells	Postitive control	Negative control
2	**PEG 3350**				
3	Coli	792µl Stock A	792µl Stock B	208µl yeast protoplats + 792µl latex bead solution	1ml yeast protop
4	Sub	792µl Stock A	792µl Stock B		
5	770	792µl Stock A	792µl Stock B		
6	767	792µl Stock A	792µl Stock B		
7					
8	**PEG 4000**				
9	Coli	792µl Stock A	792µl Stock B	208µl yeast protoplats + 792µl latex bead solution	1ml yeast protop
10	Sub	792µl Stock A	792µl Stock B		
11	770	792µl Stock A	792µl Stock B		
12	767	792µl Stock A	792µl Stock B		
13					
14	**PEG 6000**				
15	Coli	792µl Stock A	792µl Stock B	208µl yeast protoplats + 792µl latex bead solution	1ml yeast protop
16	Sub	792µl Stock A	792µl Stock B		
17	770	792µl Stock A	792µl Stock B		
18	767	792µl Stock A	792µl Stock B		
19					
20		500µl of corresponding PEG solution were added to each sample	500µl of corresponding PEG solution were added to each sample	500µl of corresponding PEG solution were added to each sample	500µl of corresp solution were ad sample

Additional dilution in samples for bacteria: 1:1.25; for yeast: 1:5

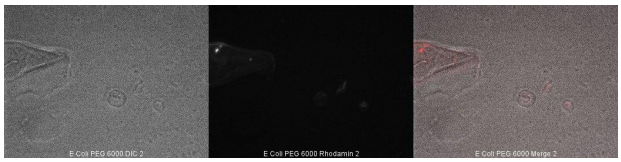
Latex bead solution: 2 drops of BD cytometer setup & tracking beads in 3ml PBS, storage at 4°C without light exposure.

After treatment and regeneration, samples were microscoped.

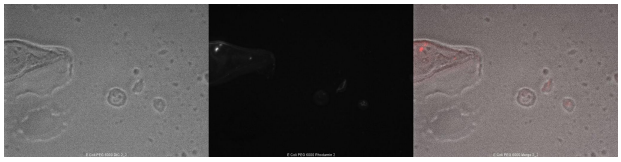
E Coli PEG 6000 Montage 1.jpg



E Coli PEG 6000 Montage 2 [+].jpg



E Coli PEG 6000 Montage 2_2 [+].jpg



All samples containing PEG 3350 and 4000 and/or B. subtilis did not contain protoplasts anymore, yet bacterial cells were intact, which could also be due to ~3-5h of unforeseeable waiting time. Only samples with PEG 6000 (able to form bigger vesicles) still contained intact protoplasts and showed possible positive results (Stock B?). Experiment will be repeated using only PEG 6000 and varying protocol conditions.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

WEDNESDAY, 27/4/16

Preparation of 25% Gly-Stocks of B. subtilis WT and DH5alpha (/w pFAB3677) and MG1655 (w/o plasmid) using a 1:1 dilution containing 50% Glycerol stock solution and each strain in corresponding media.

Plasmid miniprep of pFAB3677.

O/n culture of both M. extorquens plasmid strains in 10ml MM.

PEG fusion

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THURSDAY, 28/4/16

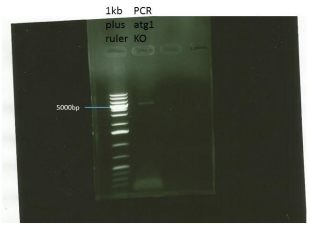
Inoculated 100ml of MG1655 for competent cells (8:30)
Prepare LB + Kan Plates

☒ Inoculate pFAB3677 again, low yields in plasmid mini prep.

PCR for ATG1 knockoutcassette:
0.5µl plasmid
10µl buffer
5µl each primer
1µl phusion polymerase
2µl dntp
26.5µl h2o

34 cycles

PCR 28.04.16.jpg



No expected PCR fragment at ~1500bp detectable, fragment at 5000bp represents the plasmid.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

FRIDAY, 29/4/16

Inoculated two times 5ml of pFAB3677.

PCR for ATG1 knockout cassette was performed again with the following conditions.

Table14

	A	B	C	D	E	F	G
1		1	2	3		PCR Program	
2	Primer fw	5µl (1:10)	5µl (1:10)	1µl (Stock)		10x	30s - 97°C
3	Primer rev	5µl (1:10)	5µl (1:10)	1µl (Stock)			30s - 54°C
4	Plasmid	0.5µl	0.5µl	0.5µl			2m40s - 68
5	DMSO	1µl	-	1µl		20x	30s - 97°C
6	DreamTaq Green PCR Master Mix (2x)	25µl	25µl	25µl			30s - 54°C
7	H20	13.5µl	14.5µl	13.5µl			3m - 68°C
8							
9	Final DNA yields in 30µl eluate	4ng/µl	30ng/µl	36ng/µl			

PCR 29.04.16.jpg

PCR product was loaded completely on 1% agarose gel (+EtBr) and extracted following the macherey&nagel gel extraction kit. DNA concentration was tested via nanodrop (table14) and stored at -20°C.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

SATURDAY, 30/4/16

- [ ] Inoculate M. extorquens (770/767) in 10 ml MM + MeOH (Mary or Steffen)

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

MONDAY, 2/5/16

- [x] Check out the different PEGs in Gerts Lab (Steffen)
- [x] Prepare 45% PEG X + 75 mM CaCl2 (sterile) (Steffen)
- [x] Make YMFM and CEN PK competent (Mary)
- [x] proceed with ATG1 KO (Basti)
- [x] Plan Experiment for Buffer conditions (?)
- [x] Prepare PEG-Experiments (check reagents, book microscope etc.) (Steffen/Patrick)
- [x] Prepare Iron Solution
- [x] Inoculate new M. extorquens (770/767) in 10 ml MM + MeOH +Tet (Steffen)
- [x] Inoculate E. Coli in 10 ml LB + Kan; Inoculate Yeast (CEN PK/YMFM) in 10 ml YPD
- [ ] check possibilities for integration cassette with fluorescent protein from Max for CEN PK
    - [x] Trafo E.coli pFAB3677

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

TUESDAY, 3/5/16

PEG 6000 with 10^8 yeast cells and testing of different stabilization buffer conditions:
Inoculation of approx. 190ml YPD with CEN.PK oD of 0.14, cells were harvested at oD of 0.6.
Preparation of spheroblasts using:

Yeast Spheroplasts (by Steffi)

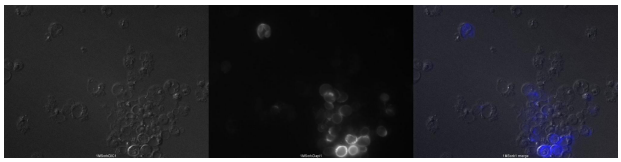
in 50ml aliquots. Two of the aliquots were pooled to a final calculated yeast concentration of oD 10 in 6ml spheroblasting medium, which was used for PEG 6000 treatment.
Since one had to be filled up with additional 10ml YPD, the other pooled aliqot had an oD of 9 in 6ml spheroblasting medium and an oD of 4.5 in 12ml spheroblasting medium. The latter was used for testing out different stabilization buffers (table 15). oD 1.04 was defined as 2x10^7 cells, 1ml of oD 4.5 should contain 9x10^7 cells.
Number of protoplasts per screen(?) was counted to estimate relative survival of protoplasts under each buffer condition. Cell number was uncountable high (but seemed to be stable).

Table15

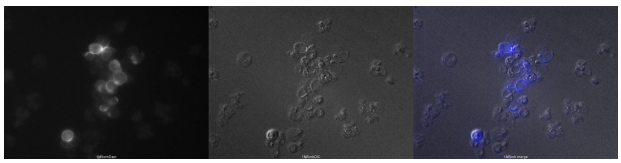
	A	B	C	D	E	F
1	**Buffer**	H2O bid	Spheroblasting medium	1M Sorbitol in YPD	0.6M KCL +0.05M KH2PO4 (pH 6)	0.7M Sorbitol in YPD (pH 5.4)
2						
3	**T(0)**					
4	Counted cells/screen	33, 60, 77, 29, 64	7, 12, 8	53, 43, 62, 41, 57	40, 55, 25	33, 39, 30, 20
5	Mean/Stdv	52.6/18.55	9/2.16	52.2/8.06	40/12.25	30.5/6.87
6						
7	**T(4h20)**					
8	Counted cells/screen	1, 0, 1, 0, 0	-	22, 18, 17, 14, 13	26, 10, 22, 22, 16	8, 17, 10, 15, 20
9	Mean/Stdv	0.4/0.49	-	16.8/3.19	19.2/5.6	12.5/3.64
10						
11	**T(24h)**					
12	Counted cells/screen	-	-	32, 30, 20, 25, 25	6, 7, 4, 4, 7	30, 37, 23, 21, 19
13	Mean/Stdv	-	-	26.4/4.22	5.6/1.36	26/6.63

10ml of each buffer were prepared, they were not sterilized

1MSorb1 montage.jpg



1MSorb montage.jpg



Test has low informative value and can not be validated in any way. Repetition with proper conditions is necessary.

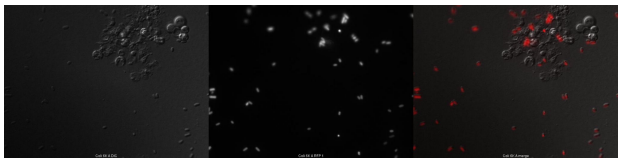
The PEG treatment was repeated with 500µl of CEN PK oD 10 stock solution, final yeast concentration should therefore be oD 5 per sample which is equivalent to 10^8 cells/ml. Additionally g-force was varried (1000x/1800x). For 2x10^9 bacterial cells/ml oD od Coli pFAB and M. ext. 770/767 was measured. Cells were diluted to final oD of 5 (1=8x10^8cells/ml). Final dilution per sample is 2.5.

pFAB oD 0.726 (1:10) = 7.26; dilution 1:1.4
770 oD 0.32 (1:10) = 3.2; no dilution. Final concentration: oD 1.6 = approx. 1.28x10^9 cells/ml
767 oD 0.39 (1:10) = 3.9; no dilution. Final concentration: oD 1.95 = approx. 1.56x10^9 cells/ml
Methylo cells were vortexed furiously before adding to sample. The PEG uptake protocol was performed using PEG 6000, 1000g or 1800g and with following samples (each with 500µl yeast + 500µl bacteria): E. coli pFAB, M. extorquens 770, M. extorquens 767. Negative ctrl 1ml yeast w/o bacteria, positive ctrl 500µl latex beads in PBS + yeast.

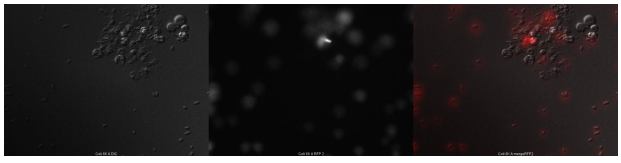
PEG induced uptake of Yeast Protoplasts

After 2h of regeneration, samples were microscoped:

Coli 6K A montage.jpg



Coli 6K A montageRFP2.jpg



Only one possible positive result was found within the E. coli sample (1000g). Treatment with 1800g showed almost complete depletion of protoplasts.

Trafo 90µl E.coli + pFAB3677: ~100 colonies
picked 15 clones, transferred to fresh LB+Kan plate, incubate o/n on 37°C
Trafo 10µl E.coli + pFAB3677: not useable.

Trafo ATG1-Knockout
since the PCR yields were super low we're using all of the deletion casette (36ng/µl in total of 60µl)

cells were prepared in prior, thaw them on 37°C for 15s.
Centrifuge 2min on 13,000g, remove supernatant.
Add transformation solution to comp cells
Transformation solution
240µl PEG
36µl 1.0M LiAc
10µl ssDNA (10mg/µl)
60µl ATG1-KO
14µl H2O

40min Heatshock on 42°C.
Centrifuge 13,000g for 30sec. Remove supernatant.
add 500µl H2O.
plate 20, 100, 200µl incubate 2-3d on 30°C.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

WEDNESDAY, 4/5/16

PEG 8000 and 20000
Protoplast formation was performed with 100ml YPD inoculated with CEN PK and harvested at oD 0.5. After pooling, 5ml of cells are concentrated to oD 10.

Yeast Spheroplasts (by Steffi)

For resuspending, the 0.6M KCL + 0.05M KH2PO4 (pH 6) was used.

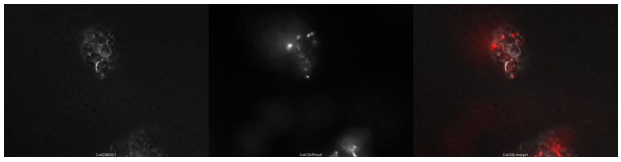
PEG protocol was performed with the same conditions as the day before (500µl yeast + 500µl bacteria). For right bacterial cell number, oD was measured before:
pFAB oD 0.7 (1:10) = 7; dilution 1:1.4
770 oD 0.44 (1:10) = 4.4; no dilution. Final concentration: oD 2.2 = approx. 1.76x10^9 cells/ml
767 oD 0.43 (1:10) = 4.3; no dilution. Final concentration: oD 2.15 = approx. 1.72x10^9 cells/ml
Methylo cells were vortexed furiously before adding to sample.

PEG induced uptake of Yeast Protoplasts

The following samples were examined: E. coli pFAB, M. extorquens 770, M. extorquens 767, negative ctrl (1ml yeast protoplasts w/o bacteria).

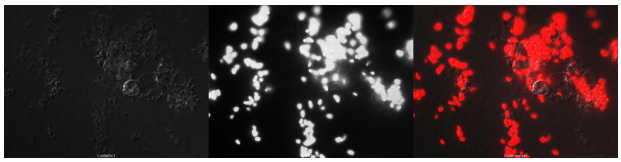
Coli20k montage [+].jpg

Coli20k montage1 [+].jpg



One positive result was found in E. coli sample using PEG 20000. In all other samples low yields of protoplasts were retained and no positive results could be seen.

Coli8k montage1.jpg



Example of Coli in yeast lysate using PEG 8000. Most other samples look similar.

Transformation of pFABq (phpNTI) in NEB Tubro
used 1µl of plasmid.
Transformation of Interlab Study constructs.
Tubes Neg. Con. & Test Device 2 were empty. wrote HQ.

Inoculate MG1655+pFAB3677 in 5ml of LB+Kan for glystock

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

THURSDAY, 5/5/16

Preparation of new LB-Cam plates

Glystock of MG1655+pFAB3677 clone 1

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

FRIDAY, 6/5/16

PCR of ATG1-KO again

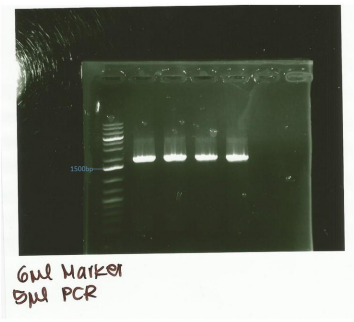
Table16

	A	B
1	MM (4x)	PCR
2	20µl Primer fwd	5µl Primer fwd
3	20µl Primer rev	5µl Primer rev
4	2µl Plasmid	0.5µl Plasmid
5	4µl DMSO	1µl DMSO
6	100µl Dream Taq Buffer (2x)	25µl Dream Taq Buffer (2x)
7	54µl H2O	13.5µl H2O

PCR Program see: Friday, 29.4 (table 14)

1%Agarose in TBE Buffer, added 20µl EtBr

PCR 06.05.16.jpg



First and last two lanes were pooled and purified via Macherey&Nagel NucleoSpin Kit. DNA yields: 1+2 (21.3g/µl); 3+4 (23.7ng/µl)

streak out competent yeast cells on YPD-Hyg and YPD plates (check survival of competent cells and Hyg-batch functionality)

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

SUNDAY, 8/5/16

Inoculating NEB+pFABq in LB+Hyg (1:500)
Preculture of yeast CEN PK

Streak out competent NEB on LB+Hyg and LB
(check survival of competent cells and Hyg-Batch functionality)

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

MONDAY, 9/5/16

50ml YPD was inoculated with CEN PK on OD600=0.2 (9:30)

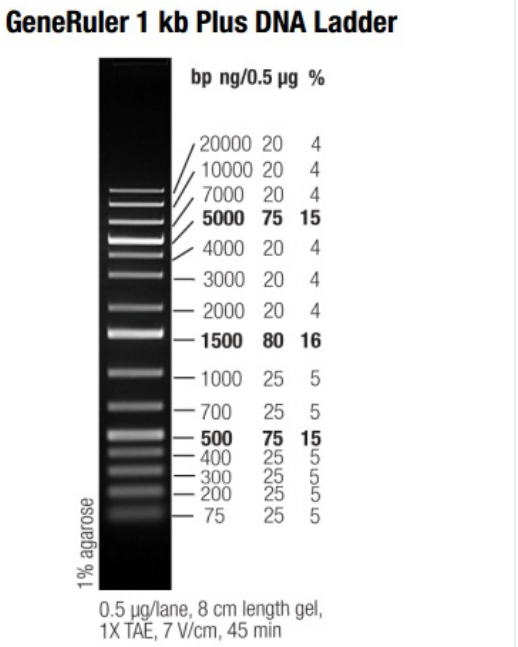
Table17

	A	B	C
1	Primer fwd	20µl	5µl
2	Primer rev	20µl	5µl
3	Plasmid	2µl	0.5µl
4	DMSO	4µl	1µl
5	Dream Taq Buffer (2x)	100µl	25µl
6	H2O	54µl	13.5µl

PCR (B) Mastermix concentrations (C) one PCR reaction.

5µl Marker, 5µl each PCR reaction
rough estimation of PCR concentration 320ng/µl (Maker Band 1500bp ~80ng/µl, Band of PCR product approx. 4x more ~320ng/µl)

Gene Ruler AG Erb.PNG



Yeast Transformation in CEN-PK

Table18

	A	B	C
1	PEG 3350	240µl	240µl
2	1M LiAc	36µl	36µl
3	ssDNA	10µl	10µl
4	plasmid	20µl	14µl
5	H2O	54µl	60µl

Transformation (B) mTurquoise Digest (C) PCR product.

40min Heatshock on 42°C. Spin down for 30s on 10,000g. Resuspend in 500µl sterile water. Plate 200µl on SC-Leu and YPD+Hyg.Incubate at 30°C.

Testing of zymolase protocol efficiency for CEN PK strain

Applying protocol with same conditions as before. To determine the living cell number, a dilution series has been made and certain dilutions were streaked out on YPD-plates. The same has been done with the cells directly after zymolase treatment, including additional SDS (same concentration as for SDS essay on 6.4.) in the medium to destroy the protoplasts and determine the number of untreatet cells. Volume of 5% SDS-stock for each sample is 333µl, filled up with either 666µl (for 1:100) or 567µl (for 1:10) spheroblasting medium.

Table19

	A	B	C	D	E	F	G
1	Dilution (Start 10^8)	10^6 (10µl from 10^8 in 990µl YPD)	10^4 (10µl from 10^6 in 990µl YPD)	10^3 (100µl from 10^4 in 900µl YPD)	10^2 (100µl from 10^3 in 900µl YPD)	10 (100µl from 10^2 in 900µl YPD)	Dilution Pro (Start 10^8
2	Streaked			X	X	X	
3	Counted cells			980	1041	72	

To visualize proto/intact yeast ratio, pictures with Calcofluor and Sytox orange were made directly after zymolase treatment. Due to cell number on plates efficiency of zymolase treatment is either 100% or intact cells get killed too.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

TUESDAY, 10/5/16

Testing of zymolase protocol efficiency for YMFM strain

Applying protocol with same conditions as before. To determine the living cell number, a dilution series has been made and certain dilutions were streaked out on YPD-Plates. The same has been done with the cells directly after zymolase treatment, including additional SDS (same concentration as for SDS essay on 6.4.) in the medium to destroy the protoplasts and determine the number of untreatet cells. Volume of 5% SDS-stock for each sample is 333µl, filled up with either 666µl (for 1:100) or 567µl (for 1:10) YPD.

Table20

	A	B	C	D	E	F	G
1	Dilution (Start 10^8)	10^6 (10µl from 10^8 in 990µl YPD)	10^4 (10µl from 10^6 in 990µl YPD)	10^3 (100µl from 10^4 in 900µl YPD)	10^3 (100µl from 10^3 in 900µl YPD)	10 (100µl from 10^2 in 900µl YPD)	Dilution Pro (Start 10^8
2	Streaked			X	X	X	
3	Counted cells			1268	313	28	

To visualize proto/intact yeast ratio, pictures with Calcofluor and Sytox orange were made directly after zymolase treatment. Only one protoplast dilution could be testet due to lack of plates.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

FRIDAY, 20/5/16

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

FRIDAY, 20/5/16

PCR for yeast Rpl5 and cPCR for E. coli YebF. The forward and reverse primers for each sample have been prepared togeter in a 1:10 dilution. For primer sequences see inventory. PCR was made with the following conditions:

1µl dNTP
10µl 5x Phusion Buffer
1µl F+R Primer
1µl (15ng) S.c. gDNA or 5µl of 1 picked E. coli colony
2µl self expressed Phusion
ad 50µl

and the following program:
10' 98°C E. coli cell denaturation; yeast samples were added. Then (30" 98°C, 1' 60°C, 2' 72°C)x30

Samples were then analyzed, only cPCR was succesful. [Bild?]

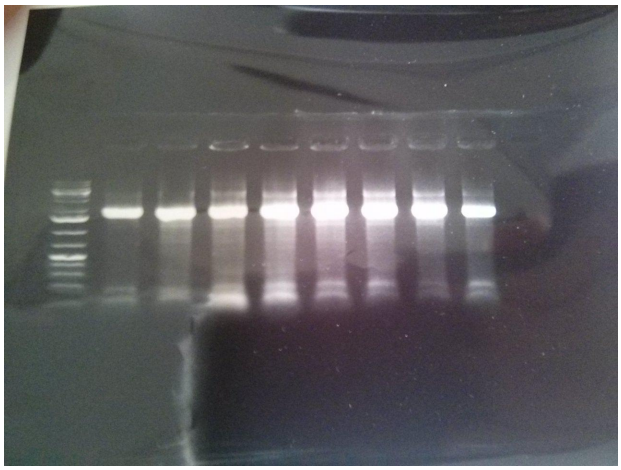
Knockouts

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-05-21 to 2016-06-07

SATURDAY, 21/5/16

ATG1-Knockout Trafo
E.coli PCR verification of ATG1-KO-plasmid (clone 1-8)

e.coli PCR for ATG1 K O Plasmid.jpg



5µl Marker
30µl PCR Product

Knockouts

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-05-21 to 2016-06-07

MONDAY, 23/5/16

ATG1-Knockout Trafo
Inoculate preculture of CEN-PK 113.71 (10ml)
prepared YPD+2%Agar

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

MONDAY, 23/5/16

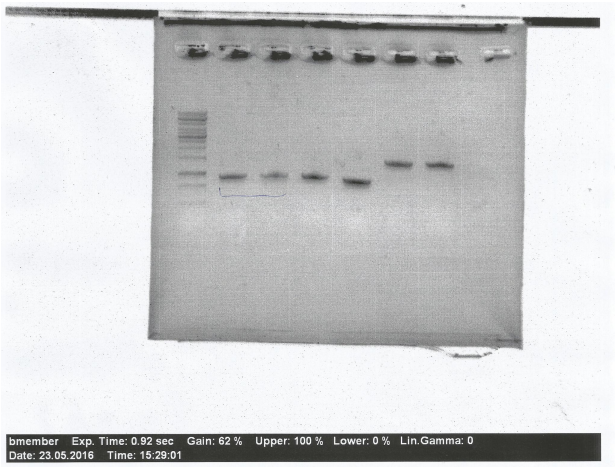
Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

MONDAY, 23/5/16

Repitition of Rpl5 PCR. Same protocol (see 20.5) was used except for denaturation step and 1.5µg/7.5µg gDNA instead of 15ng.

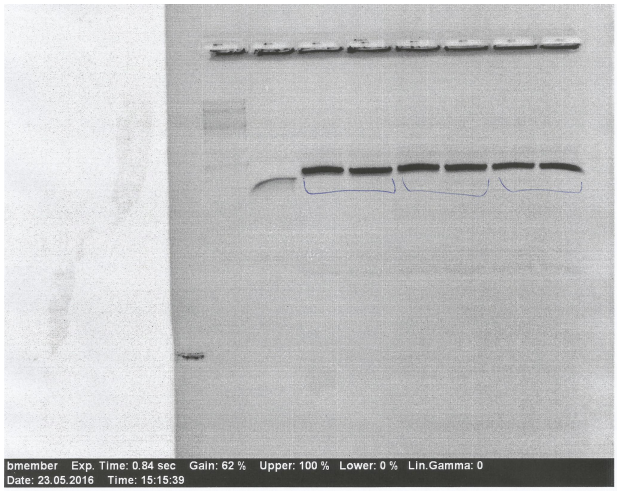
PCR 23.05 Sc rpl5 1-4.jpg



1kb generuler marker; lane 1+2 1.5µg gDNA; lane 3+4 7.5µg gDNA. Expected fragments at ~900bp
The fragments of Rpl5 were pooled and purified (Thermofischer PCR/Gel purification kit), and an overnight digestion with 1µl of each XhoI and BamHI was set up.

Amplified fragments of YebF (see 20.5) were digested with 1µl of NcoI and 1µl BamHI.

Digest N-x yebf 23.05 lane 1.jpg



d

1kb gene ruler; lane 1 digestion at ~400bp as expected.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

TUESDAY, 24/5/16

Double ligation with pET24d backbone and the amplified YebF- and Rpl5-Fragments:
0.5µl Ligase (which?)
5µl Buffer (which?)
2.1µl Vektor (diluted to 5ng/µl)
2.4µl of both inserts (1.2 per insert, both diluted to 5ng/µl) OR 2.4µl H2O as negative control
Ligation for ~10 Minutes at RT
Volumes were calculated using the iGEM ligation calculator (http://2011.igem.org/Team:UT_Dallas/ligation)

Final fusion protein should look like this:

-[NcoI]-YebF-GSGS-[BamHI]-Rpl5-6xHis-Stop-[XhoI]- with a lenght of 1201bp

Ligated vector was transformend into DH5alpha and MG1655 using following protocol:
Unfreezing at 4°C
add 5µl Ligation
10' 4°C
1' 42°C
5' 4°C
add 200µl prewarmed SOC
1h 37°C
afterwards, streak 100µl on LB-Kan plates.

Knockouts

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-05-21 to 2016-06-07

WEDNESDAY, 25/5/16

ATG1-Knockout Trafo
Inoculated main culture (50ml) on OD600=0.2, incubate at 30°C for 4h.

Harvest cells at 3,000g for 5mins.
resuspend in 25ml water, harvest at 3,000g for 5min at 20°C (2x)
resuspend in 1.2ml H2O, transfer to 1.5ml microcentrifuge tube
Spin down for 30sec at 13,000g, discard supernatant.

add Transformation mix.

Table1

	A	B
1	PEG3350	240µl
2	1M LiAc	36µl
3	ssDNA	10µl
4	plasmid	14µl
5	H2O	60µl

Heatshock on 42°C for 40mins.

plate 200µl on YPD, keep rest on 30°C o/n.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

WEDNESDAY, 25/5/16

Inoculated 200 ml CEN PK to OD 0,25

Prepare Buffers (50ml): 1M Sorbitol in YPD
0,7M Sorbitol in YPD pH: 5,4
0,6M KCl + 0,05M KH_2PO_4 pH: 6

At OD 0,5: spin down 5 min at 5000g and resuspend in 10 ml YPD

1ml was used as a microscopy Ctrl and for a dilution series: 10^3, 10^2, 10^1 cells/ml were plated. Sample in 1ml 5%SDS was plated and used as a negative ctrl. (100µl each, therefore expected cell number has to be divided by 10)
Spin down 5 min at 5000g and resuspend in 10 ml Water
Spin down 5 min at 5000g and resuspend in Softening medium + 10mM DTT
Inoculate at RT for 15 min
Spin down 5 min at 5000g
The experiment was split up into 4 samples.

1. resuspended in 5ml spheroblasting medium
2. resuspended in 5ml 1M Sorbitol in YPD
3. resuspend in 5ml 0,7M Sorbitol in YPD ph 5,4
4. resuspend in 5ml 0,6 M KCl + 0,05M KH_2PO_4 pH 6

10µl Zymolase (1U/µl) were added to each sample.
Inoculate at RT for 1h.

Prepare SDS dilution series. Corresponding medium + 20µl 5%SDS in 1ml.
Plated 10^5, 10^4, 10^3 cells/ml (100µl each, therefore expected cell number has to be divided by 10).

Fluorescent microscopy (4µl/ml Calcofluor) of 10^8 Yeast sample, Spheroblasting sample, 1M Sorbitol Sample, 0.7M Sorbitol sample, KCl Sample and Yeast + 5%SDS Ctrl. T1= 1h after Zymolase, T2=3h to determine efficiency of zymolase treatment.

Inoculated 10ml CEN PK and 10 ml MG 1655

Plated cells were counted two days later:

Table21

	A	B	C	D	E	F	G
1		Calculated cell number	10^3	10^2	10	10^5	10^4
2	Medium						
3	YPD (Yeast + ctrl)		93	9	3	-	-
4	1M Sorbitol		-	-	-	210	
5	0.7M Sorbitol		-	-	-	170	
6	KCl		-	-	-	120	
7	Spheroblasting medium		-	-	-	27	

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

WEDNESDAY, 25/5/16

Colonies could be found on Dh5alpha plates, only one on MG1655. Single colonies were picked and resuspended in 30µl H2O.
cPCR with Yebf-F and Rpl5-R primers was made to verify insert:
10µl colony
4µl HF
1µl dNTP
1µl F/R (pooled in 1:10 dilution)
1µl DMSO
2µl Phusion selfmade
1µl H2O

4 positive clones identified
[insert picture here]

Knockouts

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-05-21 to 2016-06-07

THURSDAY, 26/5/16

ATG1-Knockout Trafo

plate 200µl on YPD+Hyg of o/n probe. *achieved clones with overnight plate after 4 days

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

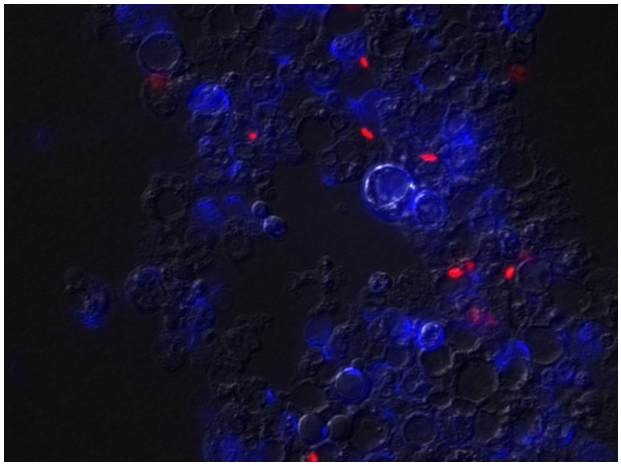
THURSDAY, 26/5/16

Preparation of PEG samples using an optimized protocol

Optimized protocol for Spheroblasts and PEG uptake

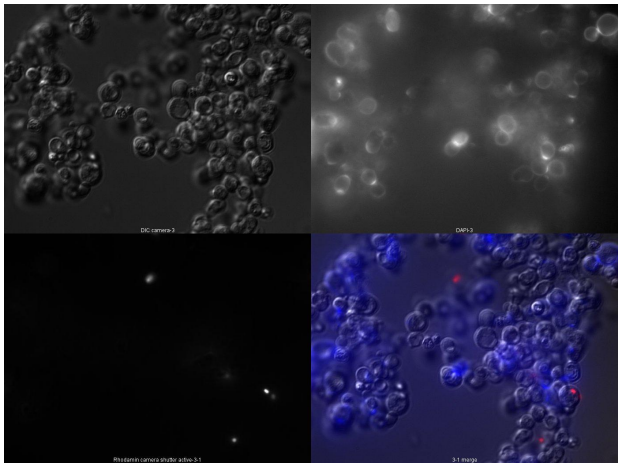
PEGs used were 20k, 20/8k (mixed 1:1) and 6k. E. coli oD 0.5 (final dilution/sample) was used.
It appears that 20k is the most efficient, but more sensitive readouts compared to micoscropy have to be done.
Additionally, agar pads for fixation kill yeasts efficiently. Micoscropy was therefore done without fixation:

20k /w agar merge.jpg



destroyed yeast on agar pad

20k /wo agar.jpg



intact yeast with positive event in bottom left corner

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

THURSDAY, 26/5/16

Prep of plasmids in DH5 alpha using thermofisher plasmid prep kit, transformation of the plasmids into BL21. Same protocol as on 24.5 was used

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

FRIDAY, 27/5/16

Ni-bead pulldown

Inoculation of BL21 colonies in 100ml LB, induction with 1mM IPTG at oD 0.9. Before induction, 200µl were taken off. 80µl of these were mixed with 20µl 5% SDS + running buffer, the remaining suspension was centrifuged, supernatant discarded and the pellet resuspended in 80µl H20, 20µl 5% SDS + buffer were added. This sample is the control before induction.
Same procedure was used 2h after induction (T+2)

For Ni-PD, the remaining ~98ml were concentrated by centrifugation (4000rpm, 10') in two aliquots.
Supernatant of one aliquot was transferred to fresh falcon and 200µl Ni-beads in EtOH were added. The pellets of both aliquots were then pooled and resuspended in Buffer A followed by homogenization with microfluidizer. The suspension was then centrifuged in the UZ at 20000rpm for 20'.
Afterwards, the supernatant was transferred to a fresh falcon and 200µl Ni-beads in EtOH were added.
Incubation of both samples with Ni-beads for 15' under constant shaking.
Centrifugation of samples at MAX 4000rpm for 15'
Supernatant was decantated, the remaining beads were resuspended in 2ml Buffer A each. After centrifugation (4000rpm, 5'), repeat this step.
Take off supernatant and resuspend remaining beads in 200µl Buffer B (=washing step). After centrifugation (4000rpm, 5'), supernatant should contain tagged protein and 80µl of each can be mixed with 20µl 5% SDS + running buffer.
Before running the SDS-Page, cell lysis for 10' on 98°C is necessary for the pellet-samples.

To determine whether the supernatant contained Rpl5-His, a SDS-Page was made (35' at 270V) and stained with coomassie.
[Picsoritdidnthappen]

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

MONDAY, 30/5/16

No protein expression could be detected with the experiment on friday. Therefore plasmid was transformed into a BL21 Rosetta strain (next day)

Knockouts

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-05-21 to 2016-06-07

TUESDAY, 31/5/16

ATG1-Knockout Trafo

picked 8 colonies, plate them on new YPD+HYG for gDNA preparation and PCR.

Yeast Lysate Growth Test
inoculated 1.5L YPD with CEN PK 113.7D

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

TUESDAY, 31/5/16

Transformation into Bl21 Rosetta
thraw chemocompetent cells on 4°C
add 1µl/2µl vector with fusion protein
10' 4°C
1' 42°C
5'4°C
add 200µl 42°C SOC
min. 1h 37°C
plate 100µl on LB-Cam-Kan plates (Cam because of Rosetta resistance)

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

WEDNESDAY, 1/6/16

- Codon optimized versions of *matB* of *Rhizobium* leguminosarium and *mae1* of *Saccharomyces pombe* were orderes at IDT.
- Recieved plasmid pNK26 that carried the bidirectional promoter region for the expression plasmid (iGEM1.1) and the backbone for the expression plasmid (iGEM1.3)

Knockouts

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-05-21 to 2016-06-07

WEDNESDAY, 1/6/16

poured YPD+Hyg plates. Hyg batches are empty.

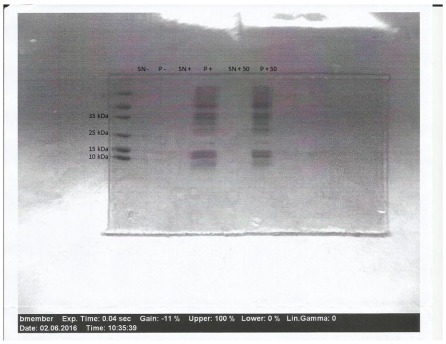
Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

WEDNESDAY, 1/6/16

Trafo succesful, inoculated colonies in 100ml with 100µl Kan.
Ni-PD was done with the same conditions as on 27.5.

Ni-PD 1.6.jpg



Excellent quality picture of the SDS-Page from the Ni-PD. SN=supernatant, P=Pellet, - before induction, + after induction, 50 = whole pulldown

Low expression yields could be detected, more in the cell lysate than in the supernatant (for the two 50 lanes). Experiment will be repeated with a time of ~20h after induction

Synechocystis PCC 6803

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-06-01 to 2016-06-13

WEDNESDAY, 1/6/16

Prepared 1L of BG11 from Stock solutions (provided by Jan).

Culture we recieved from MCC is eventually contaminated, clean up of PCC6803 liquid culture.
Spun down approx. 12ml at 4000rpm
washed in ddH2O (osmotic pressure should kill most contaminants.)
added 5ml BG-11, resupended cells

streak out 35µl of washed/unwashed PCC6803 on BG11-plates
inoculated 200ml of BG-11 media with 2ml of PCC6803 washed/unwashed
incubate on 25°C /w 0.2% CO2 for 3-4 days.

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

THURSDAY, 2/6/16

Amplification of fragment iGEM1.1 of the expression plasmid using pNK26 as template.

- 1µl template
- 2.5µl NK367 primer
- 2.5µl NK368 primer
- 1µl dNTPs
- 10µl 10x phusion high fidelity buffer
- 32.5µl H2O
- 1µl Phusion polymerase.

Estimated band lenghts 1405bp.
Amplification of this fragment was successful and cleaned from PCR reaction.

Amplification of fragment iGEM1.3 of the expression plasmid using pNK26 as template

- 1µl template
- 2.5µl NK365 primer
- 2.5µl NK366 primer
- 1µl dNTPs
- 10µl 10x phusion high fidelity buffer
- 32.5µl H2O
- 1µl Phusion polymerase.

Estimated band lenghts 5970bp.
Amplification of this fragment was successful and cleaned from PCR reaction.
.

Knockouts

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-05-21 to 2016-06-07

THURSDAY, 2/6/16

Yeast Lysate Growth Test: Lysate Preparation

harvested SNK76 at OD600= 10.9 from tuesday in ultracentrifuge 6.000g for 30min.

added 80ml icecold water to resuspend cells (OD600= 204.4)
homogenized cells in bead beater 10x for 10sec at 6000rpm with 2min cooling in between each step. (5ml beads, 10ml cellsuspension)
lysate (supernatant) was pooled in 50ml falcons after centrifuging 5min at 6.000g (4°C).
lysate was centrifued again in ultracentrifuge (100.000g) for 1h to seperate cellwall/membrane from cytosolic part.

lysate was sterile filtred and stored at 4°C.

Yeast Lysate Growth Test: FACS

probes for FACS were as follows, inoculated to an OD600=0.2

Table2

	A	B	C	D	E	F	G
1	**Medium**	**Yeast Lysate**	**5% SDS**	**Tethering Buffer**	**PBS**	**BG-11**	**LB**
2		Yeast Lysate + E.coli MG1655	5% SDS + E.coli MG1655	Tethering Buffer + E.coli MG1655	PBS + E.coli MG1655	BG-11 + PCC6803	LB + E.coli MG1655
3		Yeast Lysate + PCC6803	5% SDS + PCC6803	Tethering Buffer + PCC6803	PBS + PCC6803		
4		Yeast Lysate + M. ex 770	5% SDS + M. ex 770	Tethering Buffer + M. ex 770	PBS + M. ex 770		

FACS Data will be implemented by Basti.

Yeast Lysate Growth Test: plate reader

still contamination in pcc6803.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

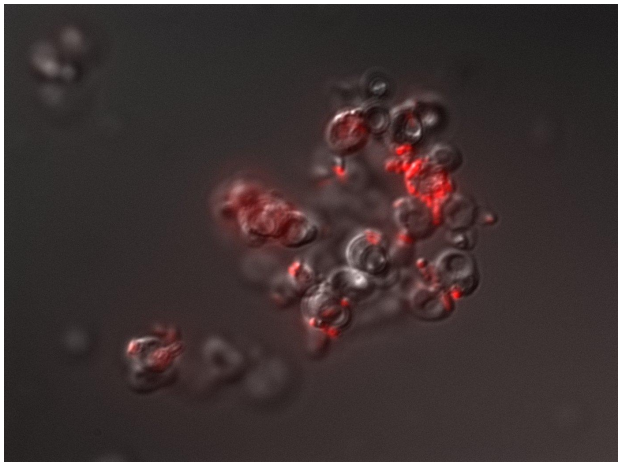
THURSDAY, 2/6/16

PEG method was repeated with YMFM strain to test whether it is suitable too. Yeast cells were also diluted and plated (10^3, 10^2 and 10 cells/ml), as well as spheroblasts (10^5, 10^4 and 10^3 cells/ml in KCl + 20µl 5% SDS) were plated

Optimized protocol for Spheroblasts and PEG uptake

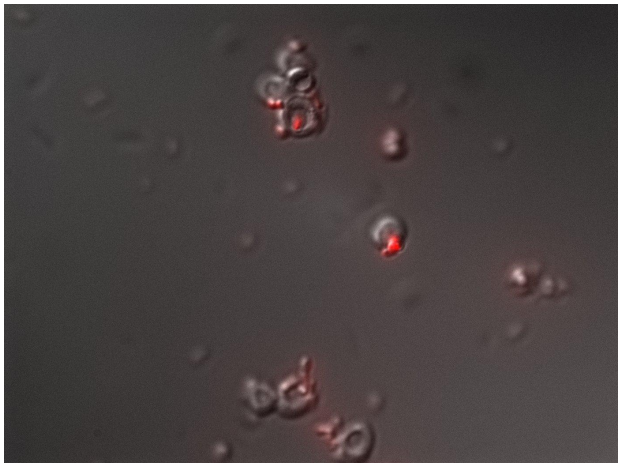
KCl medium had to be used as spheroblasting medium. E. coli oD of 1 in final sample (=8+10^8cells/ml)
Additionally, cells were sonificated for 15" to get rid of clumps.
PEGs used this time: 20k, 20k/8k (1:1 mixture) and 8k.

merge wo dapi.jpg



lysis due to dividing coli

6 merge wo dapi.jpg



example of positive event on the upper side

Some succesful events could be detected, mostly with samples containing PEG. Surprisingly some cells seem to have bursted because of coli division inside. Sonification does not seem to have an effect.
Apparently the YMFM plasmid mTurquoise is detectable on the CFP-channel, not on the DAPI-channel. No merge with yeast staining.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

THURSDAY, 2/6/16

Inoculation of 100ml LB ROS1 with 100µl Kan. After oD reached 0.5, induction with 0.1µM IPTG (10µl 1M). Culture was placed at 30°C incubator overnight.
300µl sample were taken and centrifuged. Then again, 80µl of the supernatant and 80µl of the resuspended pellet were taken, mixed with 20µl SDS running buffer and kept on -20°C.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

FRIDAY, 3/6/16

Cells were harvested at oD 1.5. A sample like on 2.6 was taken and kept at RT.
For Ni-PD, the protocol was followed as on 27.05.

Synechocystis PCC 6803

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-06-01 to 2016-06-13

FRIDAY, 3/6/16

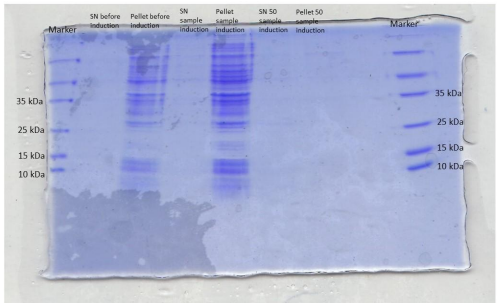
plates showed many contaminants. washed again for 5min once, twice, and once for 10 mins.
spin down for 4 min at 2500g.
inoculated 50ml of fresh media with 2.5ml of washed culture.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

SATURDAY, 4/6/16

yebf-rpl5 3.6.jpg



Marker seems to be running in all lanes. Due to bad resolution of the picture, the small expected band at ~34kDa on the SN50 lane can not be seen. It was cut out for further analysis with mass spec.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

MONDAY, 6/6/16

PEG for M. extorquens and Synechocystis sp. PCC 6803. For Synechocystis, an oD_{750} of 0.5 correlates with an approximate cell number of 4*10^7. (http://unitedscientists.org/labs/norway/NTNU/PhotoSynLab/wiki/table-of-contents-2/organisms/cell-number-optical-density).
Concentration to an calculated oD of 1.5 should then correlate with a cell number of 10^8 which will be used for the experiment.

Optimized protocol for Spheroblasts and PEG uptake

Controls are 1ml yeast spheroplasts in 20k PEG and 1ml PCC6803 in 20k PEG.

Synechocystis PCC 6803

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-06-01 to 2016-06-13

MONDAY, 6/6/16

investigated cultures under light microscope. still contaminants.
washed again once for 30mins in ddH2O and twice for 5mins in ddH2O.

incubate at 25°C.

Knockouts

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-05-21 to 2016-06-07

TUESDAY, 7/6/16

Yeast Lysate Growth Test: plate reader

volume/well: 100µl
starting OD600/750 for each strain: 0.05
Measurements every 10 mins for 100 cycles.

Table3

	A	B	C	D	E	F	G
1		1	2	3	4	5	
2	A	H2O	YL	5%SDS	MM+Tet	LB+Kan	BG-11
3	B	YL + M. ex 770	YL + M. ex 770	YL + M. ex 770	YL + MG1655	YL + MG1655	YL + MG1
4	C	MM+Tet M. ex 770	MM+Tet M. ex 770	MM+Tet M. ex 770	LB-Kan + MG1655	LB-Kan + MG1655	LB-Kan + MG1655
5	D	5% SDS + MG1655	5% SDS + MG1655	5% SDS + MG1655	5% SDS + MG1655	5% SDS + MG1655	5% SDS + MG1655
6	E	PBS + MG1655	PBS + MG1655	PBS + MG1655	PBS + MG1655	PBS + MG1655	PBS + MG
7	F	Tethering + MG1655	Tethering + MG1655	Tethering + MG1655	Tethering + MG1655	Tethering + MG1655	Tethering + MG1655

Synechocystis PCC 6803

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-06-01 to 2016-06-13

WEDNESDAY, 8/6/16

Preparation of BG-11 liquid media + plates and stock solutions.

Table1

	A	B	C	D	E	F
1	**BG-11 (1L)**	**100x BG-FCS (1L)**	**1000x Fe ammonium citrate (50ml)**	**Trace minerals**	**1000x Na2CO3 (50ml)**	**1000x K2HPO4 (50ml)**
2	10ml 100xBG-FCS	149.58g NaNO3	0.3g Fe ammonium citrate	2.86g H3BO3	1g Na2CO3	1.52g K2HPO4
3	1ml 1000x Fe ammonium stock	7.49g MgSo4 * 7 H2O		1.81g MnCl2 * 4H2O		
4	1ml 1000x Na2CO3	3.6g CaCl2 * 2H2O	**dissolve in 50ml, store at 4°C**	0.222g ZnSO4 * 7H2O	**dissolve in 50ml, store at 4°C**	**dissolve in 50ml, store at 4°C**
5	1ml 1000x K2HPO4	0.6g (sodium) Citrate	**sterile filtrated.**	0.39g Na2MoO4 * 2H2O	**sterile filtrated.**	**sterile filtrated.**
6	20ml 20mM HEPES KOH (pH 8.0)	1.12ml Na-EDTA, 250mM, pH 8.0		0.079g CuSO4 * 5H2O		
7	967ml ddH2O	100ml Trace Mineral solution		0.0494g Co(NO3)2 * 6 H2O		
8	for plates add 1.5% agar	**dissolve in 1L, store at 4°C**		**dissolve in 1L, store at 4°C**		
9	**autoclaved.**			**sterile filtrated.**		
10						

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

THURSDAY, 9/6/16

PEG for E. Coli, M. extorquens, Synechocystis sp. PCC6803.
Instead of recovery in regeneration medium YNB Medium with 0.7M Sorbitol and pH 5.4 was prepared

0.67g YNB w/o AA
0.5g glucose
12.75g Sorbitol
pH: 5.4

Optimized protocol for Spheroblasts and PEG uptake

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

FRIDAY, 10/6/16

- Amplification of the codon optimized mae1 from synthesized nucleotide sequence.
    1µl template
    2.5µl NK371 primer
    2.5µl NK372 primer
    1µl dNTPs
    10µl 10x phusion high fidelity buffer
    32.5µl H2O
    1µl Phusion polymerase.

Estimated band lenghts at 1406bp
Amplification was unsuccessful. No bands were visible.

- Amplification of the codon optimized mae1 from synthesized nucleotide sequence.
    1µl template
    2.5µl NK371 primer
    2.5µl NK372 primer
    1µl dNTPs
    10µl 10x phusion high fidelity buffer
    32.5µl H2O
    1µl Phusion polymerase.

Estimated band lenghts at 1406bp
Amplification was unsuccessful. No bands were visible.

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

MONDAY, 13/6/16

Topo cloning using blunt end PCR product of mae1 and matB into blunt end topo vector following instruction of the kit.
The reaction was transformed into NEB Turbo and plated an LB Amp plate.

Synechocystis PCC 6803

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-06-01 to 2016-06-13

MONDAY, 13/6/16

dilute cultures. plates show still contamination. need antibiotics against gram neg. bacteria.

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

TUESDAY, 14/6/16

Several colonies grew on the LB Amp plates.

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

THURSDAY, 16/6/16

Repeating PCR from 13.06. with different annealing temperatures.
Again PCR did not show bands at estmated lenghts.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

THURSDAY, 16/6/16

PEG for E. coli and PCC6803. Afterwards fixation with PFA
oD for Coli: 1.1 in final sample. oD for PCC6803: 1.2 in final sample. A 5:1 mixture of PEG 20000 : 3350 was used which makes it more soluble.
To try whether an incubation with bacteria + PEG only affects the efficiancy, the yeast cells were added after step 17 of the protocol. The other samples were treated as usual.

Optimized protocol for Spheroblasts and PEG uptake

After the PEG induced uptake, each sample was split into two 750µl aliquots, one of each for PFA fixation for better micoscropy pictures using the following protocol:

PFA Fixation for yeast

Controls are good, bacteria are not fixated and the samples are dead.
PICTURES soon

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

FRIDAY, 17/6/16

Antibiotics titration:

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

FRIDAY, 17/6/16

Primers. Finally. Amplification of ß-glucuronidase from E. coli possible
cPCR with primers (diluted 1:10 in an F+R aliquot, 80µl H2O+10µl each primer)

OLD BamHI-GUS-F

OLD GUS-Stop-XhoI-R

as follows:
1µl DMSO
1µl F+R
2µl dNTP
5µl colony (picked and resuspended in 30µl H2O)
5µl Taq-Buffer (10x)
0.25µl Taq-Pol (Roboklon, 5U/µl)
ad 35.75µl H2O

and the following PCR setup:
10' 95°C initial denaturation
30" 95°C denaturation |
40" 65°C annealing | x34
90" 72°C elongation |
samples were stored at 4°C and will be tested tomorrow

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

SATURDAY, 18/6/16

PCR was not successful, neither was a retry with a) 0.1µM each primer, 5' initial denaturation or b) 0.25µl plant optimized GUS template instead of colony.
Primers are interestingly not completely matching the MG1655 gusA (ß-glucuronidase) gene, a mysterious template for primer design was used. Must be retried with new primers.

Western Blot
Done with the prepared samples of the 3.6.16
Run SDS PAGE (prepared gel by Sonja) at 100V 50mA for stacking and 150V 50mA for resolving gel.
Problems: Stacking gel might be too small for higher amounts of loading. Missing loading tips/syringes.

Loading:

Table1

	A	B	C	D	E	F	G
1	Marker	Pellet not induced	SN not induced	Pellet induced	SN not induced	Pellet 50 induced	SN 50 indu
2	5µl	25µl	25µl	13µl	13µl	25µl	25µl

Blot gel on Nitrocellulose membrane at 250V for 1,5h
Block Membrane with 5% milk over night.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

SUNDAY, 19/6/16

Prepare Anti Tetra His Antibody. 1:1000 in 5% milk (stored at 4°C)
2h at RT in 1st Ab

Wash 3x 5 min in PBST

1h in Anti Mouse Igg + HRP 2nd Ab (1:2500)

Wash 3x 5 min in PBST

Use ECL reagant (less luminol as the kit was nearly empty)
Develop blot
stored membrane in new membrane box in the cupboard above the bench.

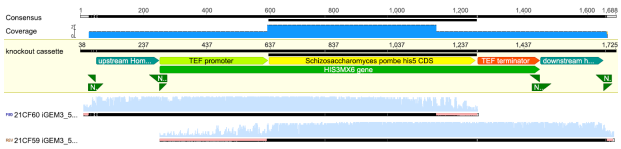
Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

WEDNESDAY, 13/7

Sequencing of 4 mae1 clonies and 4 matB colonies via GATC
Sequencing of iGEM2 (ACC1 KO) (MK5.1 & MK5.8)

ACC1 KO Sequencing.tif



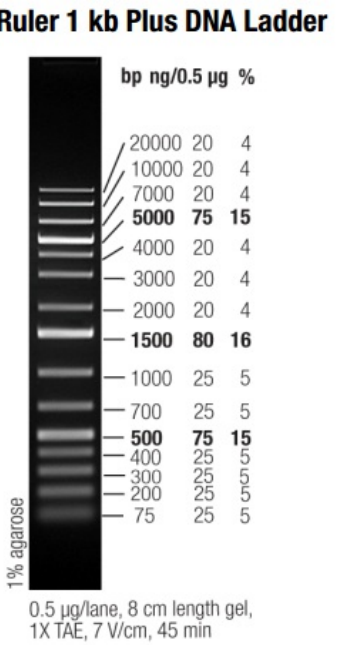
Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

FRIDAY, 15/7

Got Plasmids pFosbeta, pJunbeta, pHebeta from Victor de Lorenzo.

Gene_Ruler_AG_Erb.png



because i always search for it.

Cell Aggregation Assay

Introduction

Protocol is used as described in Veiga et al, 2003. Testing of Plasmids provided by them.

Materials

- LB w/o glucose
- 0.5M IPTG (stock solution)
- culture tubes

Procedure

1. Grow plasmids seperately to OD600 **0.5**.
2. Induction with **0.5mM** IPTG for **3h** at 37°C, shaken. (1:1000)
3. 5ml Aliquots can be mixed (pJun/pJun, pFos/pJun, pFos/pFos) - transfer to 10ml tubes, cultivate **without shaking**.
4. 100µl samples **every 20min**s are taken from top of the tube and OD is measured.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

SUNDAY, 17/7

Inoculated in 20ml LB+CAM each
Inoculated pILS7 in 20ml LB+CAM

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

MONDAY, 18/7

Minipreps from pFosb, pJunb, pHeb and pILS7

BHUM2200 Trafo in NEB Turbo and MG1655 on LB+AMP
2 colonies on NEB plate, no colonies on MG1655.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

TUESDAY, 19/7

- ☒ Digest of pFosb, pJunb, pILS7 w/ NotI
- ☒ Dephosphorylation of pILS7
- ☒ Gel extraction of pJun, pFos
- ☒ Ligation of pJun w/ pILS7 and pFos w/ pILS7

Digest: NotI

NotI Digest

	A	B	C	D	E
1	**2µl pJunb**	**2µl pFosb**	**10µl pHeb**	**10µl pILS7**	**1µg Plasmid**
2	5µl CutSmart	5µl CutSmart	5µl CutSmart	5µl CutSmart	**5µl CutSmart Buffer**
3	1µl NotI	1µl NotI	1µl NotI	1µl NotI	**1µl NotI**
4	42µl H2O	42µl H2O	34µl H2O	34µl H2O	**up to 50µl H2O**

on 37° for 2h.

Gel-Extraction worked poorly, but still:

pJunb 5.8ng/µl
pFos 8.6ng/µl
pILS7 21.6ng/µl

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

WEDNESDAY, 20/7

Dephosphorylation w/ Fast-AP (NEB)

pILS7 (all total 216ng) 15µl
10x Buffer 2µl
Fast AP 0.5µl
water 2.5µl
total 20µl incubated at 37°C for 10', heat inactivation at 75°C for 5'

Ligation

Table1

	A	B	C	D	E
1	**Vector (Neg Con)**	**Vector + pJunb**	**Vector + pFosb**	**Neg Con**	
2	2µl	2µl	2µl	2µl	**pILS7 (21.6ng/µl)**
3	-	10µl	10µl	-	**pFos (5.8ng/µl) or pJun (8.4g/µl)**
4	2µl	2µl	2µl	2µl	**10x Buffer**
5	15µl	5µl	5µl	17µl	**H2O**

incubate at room temperature fo 1.5h

Trafo in NEB Turbo.

5µl Ligation
30' ice
90" heatshock 42°C
cool on ice, add 800µl LB
120' recovery 37°C
plate all on LB-CAM.
incubate o/n at 37°C.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

THURSDAY, 21/7

Trafo in NEB Turbo.

neg controls (water only, dephos. vector only) showed no colonies.
pJUN ~15 colonies (lowest concentration after GelEx)
pFOS ~ 200 colonies

for analytical digest
picked five clones each, inoculated in 7ml LB+CAM.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

FRIDAY, 22/7

Minipreps of four clones each.

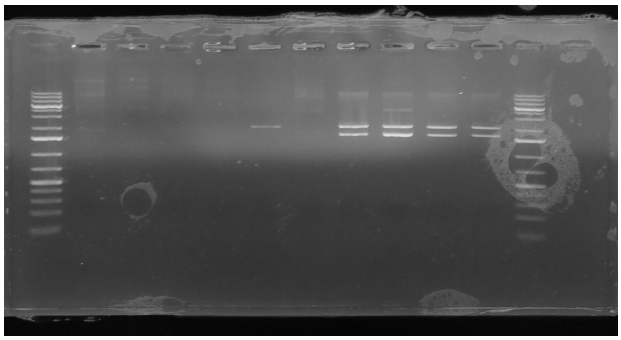
Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

MONDAY, 25/7

analytical digest of clones w/ NotI

analytical digest NotI.JPG



lanes (ltr) M, pJUN1, 2, 3, 4, pILS7bb, pJUN nondigested, pFos 1, 2, 3, 4, M

positive clones: pFos1, 2, 3, 4. can get send to sequencing

inoculated pJUN clones 5, 6, 7, 8 in 5ml LB+CAM.

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

MONDAY, 25/7

Inoculated the received E. coli strains with the plasmids pMevT (Cm), pMevB(Tet), pMBIS (Tet), pLac3390E4 (pSOE4?) (Cm) in 5ml LB + 5µl corresponding antibiotic. Inoculation over night at 30°C.

Stored plates over night at 30°C.

Cell Aggregation Assay

Introduction

Protocol is used as described in Veiga et al, 2003. Testing of Plasmids provided by them.

Materials

- LB w/o glucose
- 0.5M IPTG (Stock solution)
- culture tubes

Procedure

1. Grow plasmids seperately to OD600 **0.5**.
2. Induction with **0.5mM** IPTG for **3h** at 37°C, shaken. (1:1000)
3. 5ml Aliquots can be mixed (pJun/pJun, pFos/pJun, pFos/pFos) - transfer to 10ml tubes, cultivate **without shaking**.
4. 100µl samples **every 20min**s are taken from top of the tube and OD is measured.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

TUESDAY, 26/7

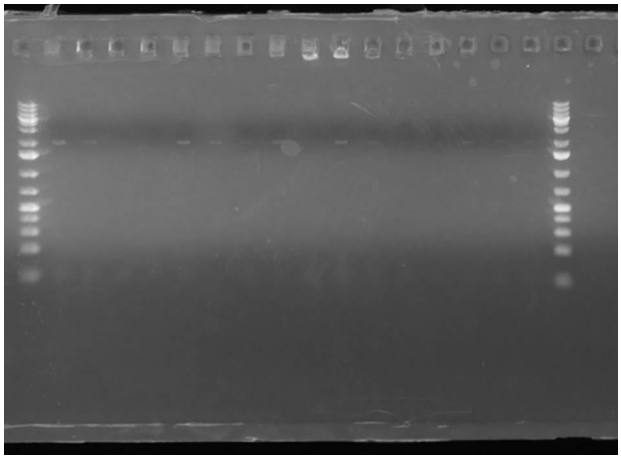
Miniprep of clones.
analytical digest of clones w/ NotI
all negative.

colonyPCR with 16 clones.
10µl DreamTaq Buffer
8µl H2O
2µl Primer (VR, VF2. 10pmol each)

Table2

	A	B	C	D
1	**98°C**	initial Denaturation	**5'**	
2	**95°C**	Denaturation	**30"**	|
3	**55°C**	Annealing	**45"**	| x30
4	**72°C**	Extension	**90"**	|
5	**72°C**	final Extension	**5'**	

colonyPCRpJUN260716.jpg



positive clones in lanes 1,5,6,10,11,14.
will send clone 9 (lane 2) for sequencing.

Dependencies

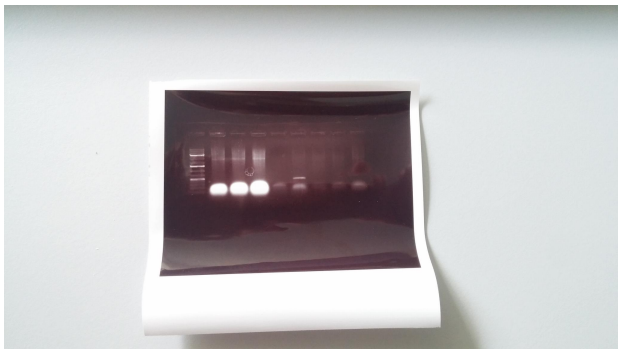
Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

TUESDAY, 26/7

Colony PCR of 4 mae1 colonies and 4 matB colonies using M13 primers
total: 20µl
taqDream: 10µl
H2O: 8µl
Primer: 2x1µl

1. 5min - 95°C
2. 1min - 95°C
3. 1min - 56°C
4. 1min - 72°C (go to 2 x30)
5. 5min - 72°C

20160727_113843.jpg



No positives. Short band at around 100bp suggests that the topo vector religated.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

TUESDAY, 26/7

Two colonies from MG1655 on LB were picked and resuspended in 30µl H2O

New cPCR for YebF and GusB (NEW primers) using the following conditions (each PCR was performed with both picked colonies):
1µl DMSO
1µl fw + rev primer (1:10)
5µl each colony
1µl dNTP 10mM
10µl 5x Phusion Buffer HF
1µl Phusion Pol
31µl H2O

10' 98°C
30" 98°C|
1' 60°C | x30
2' 72°C |
10' 72°C

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

WEDNESDAY, 27/7

Minipreps of colne9, 13, 19.
clone 9 is send for sequencing.

Dependencies

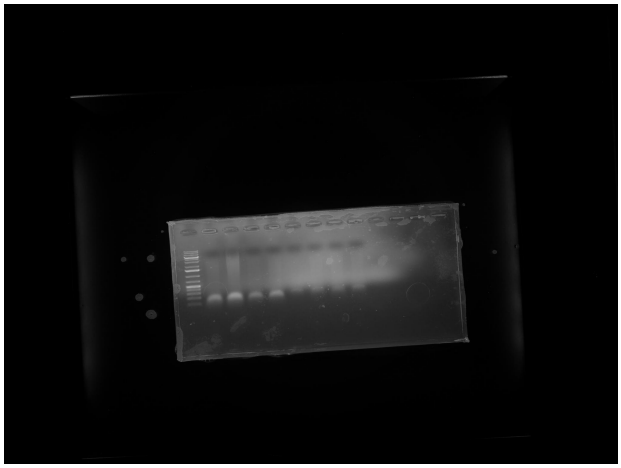
Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

WEDNESDAY, 27/7

Repeating colony PCR with 8 new colonies. Decreasing annealing temperature and annealing time to avoid unspecific amplifications

1. 5min - 95°C
2. 30sec - 95°C
3. 30sec - 50°C
4. 1min - 72°C (got to 2 x30)
5. 5min - 72°C

P1000141.JPG



No Positives.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

WEDNESDAY, 27/7

Digestion of pET28b, Yebf and GusB:

Table2

	A	B	C
1	pET28b	YebF	GusB
2	1µl NcoI 1µl XhoI 3µl Vector (287ng/µl) 5µl Cutsmart 5x Buffer 42µl H2O	1µl NcoI 1µl BamHI 30µl Fragment (22.5ng/µl) 5µl Cutsmart 5x Buffer 13µl H2O	1µl BglII 1µl XhoI 30µl Vector (33.4ng/µl) 5µl Buffer 3.1 5x Buffer 13µl H2O

for 1h at 37°C.
Afterwards heat inactivation for 20 min on 80°C. Purification of fragments was performed using the zymo DNA clean & concentrate kit 5.
Ligation of all three fragments (according to igem texas ligation calculator):
50ng/µl dilution of each fragment.
1µl 10x buffer
1µl T4 ligase
3µl diluted pET28b (or leftovers from pET24d)
2.5µl diluted YebF, 2.5µl diluted GusB (or 5µl water as ctrl)

for 10min at RT.

Transformation with ligated vectors was performed using competent NEB Turbo cells from stock
Unfreezing at 4°C
add 5µl Ligation
10' 4°C
1' 42°C
5' 4°C
add 200µl prewarmed LB
1h 37°C
afterwards, streak 200µl on LB-Kan plates. #belive

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

THURSDAY, 28/7

X-Gal Plates for Blue White Test:
10ml lb Agar
100µl X-Gal
100µl IPTG
10µl Amp

Plating remaining clonies to identify religated Topo-Vectors.

All remaining colonies showed blue color.
Topo-cloning was unsuccesful and needs to be repeated.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

THURSDAY, 28/7

Trafo not succesful, repition under the same conditions except following changes:
Digestion of pET28b, Yebf and GusB:

Table3

	A	B	C
1	pET28b (30µl full plasmid prep)	YebF (30µl full prep)	GusB (30µl full prep)
2	1µl NcoI 1µl XhoI 3µl Vector (26ng/µl) 5µl Cutsmart 5x Buffer 13µl H2O	1µl NcoI 1µl BamHI 30µl Fragment (22.5ng/µl) 5µl Cutsmart 5x Buffer 13µl H2O	1µl BglII 1µl XhoI 30µl Vector (33.4ng/µl) 5µl Buffer 3.1 5x Buffer 13µl H2O

for 1h at 37°C.
Afterwards heat inactivation for 20 min on 80°C. Purification of fragments was performed using the zymo DNA clean & concentrate kit 5.
Ligation of all three fragments (according to igem texas ligation calculator):
10ng/µl dilution of each fragment.
1µl 10x buffer
1µl T4 ligase
3µl diluted pET28b
2.5µl diluted YebF, 2.5µl diluted GusB (or 5µl water as ctrl)

for 10min at RT.

Transformation with ligated vectors was performed using competent NEB Turbo cells from stock
Unfreezing at 4°C
add 5µl Ligation
10' 4°C
1' 42°C
5' 4°C
add 200µl prewarmed LB
1h 37°C
afterwards, streak 200µl on LB-Kan plates.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

FRIDAY, 29/7

Trafo succesful. To verify insert, cPCR was performed:
5µl colony (resuspended in 30µl H2O)
2µl 10mM dNTP
1µl Yebf fw + GusB rev (1:10)
0.25µl optiTaq
5µl Buffer C
ad 50µl H2O

5' 98°C
30'' 95°C|
1' 56°C |x30
2' 72°C |
10' 72°C

cPCR not succesful, will repeat with Phusion

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

MONDAY, 1/8

Inoculate MG1655, BW25113, BW25113 deltaompT, UT5600 for Transformation

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

MONDAY, 1/8

Performed cPCR under following conditions
5µl colony (resuspended in 30µl H2O)
1µl 10mM dNTP
1µl Yebf fw + GusB rev (1:10)
1µl Phusion
10µl Phusion Buffer 5x
ad 50µl H2O

10' 98°C
30" 98°C|
1' 60°C |x30
2' 72°C |
10' 72°C

Still no success. Will repeat again due to lack of purified DNA fragments.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

TUESDAY, 2/8

Competent cells of MG1655, BW25113, BW25113 deltaompT, UT5600 -
Transformation of pJUN, pFOS, pUC19 into competent cells.
used 2µl Plasmid each.
30mins ice, 1.5min heatshock, 2h recovery 37°C
plated 200µl

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

WEDNESDAY, 3/8

Trafo of MG1655 did not work. pJUN Trafo in BW25113deltaompT did not work.
Repeated Trafo of those.
Rest worked fine. (~50-200 colonies)

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

MONDAY, 8/8

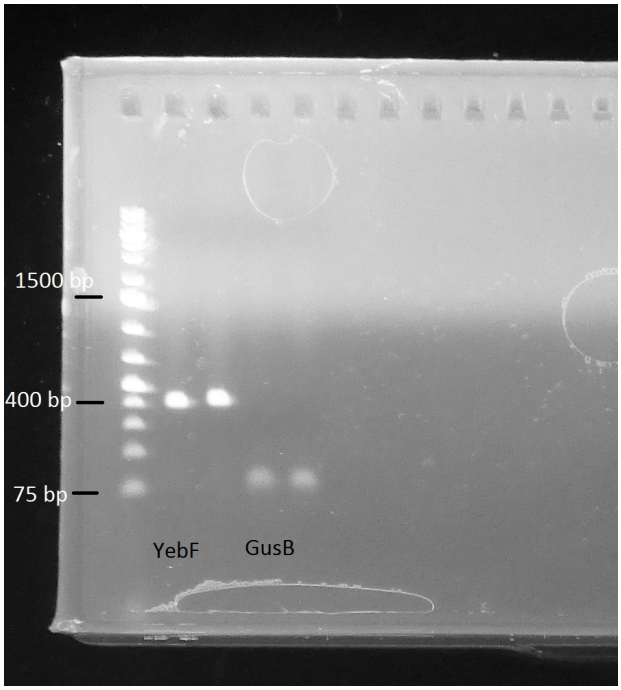
One colonie from MG1655 on LB was picked and resuspended in 30µl H2O

Two cPCR for YebF and and two cPCR for GusB (NEW primers) using the following conditions (each PCR was performed with picked colonie):

1µl fw + rev primer (1:10)
5µl each colony
1µl dNTP 10mM
10µl 5x Phusion Buffer HF
0,5µL Phusion
32,5µl H2O

10' 98°C
30" 98°C|
1' 60°C | x31
30" 72°C (for YebF) | 1' 72°C (for GusB)
10' 72°C

PCR_1.png



PCR for GusB didn't work. Will repeat with new diluted primers and same conditions as above.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

TUESDAY, 9/8

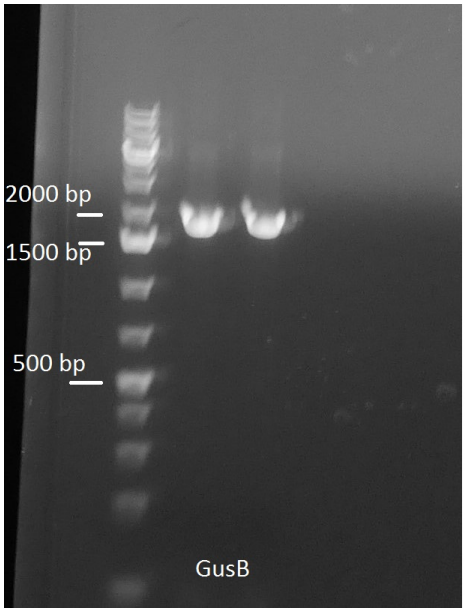
Trafo of BW25113deltaompT did work ~ 200 colonies.
MG1655 did not work.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

TUESDAY, 9/8

PCR_GusB.png



The same PCR-Product of GusB was mixed together and purified using the zymo DNA clean & concentrate kit 5.

Digestion of pET28b, YebF and GusB:

Table4

	A	B	C
1	pET28b (25µl full plasmid prep)	YebF	GusB
2	1µl NcoI 1µl XhoI 25µl Vector (44ng/µl) 5µl Cutsmart 10x Buffer 18µl H2O	1µl NcoI 1µl BamHI 7µl Fragment (121,4ng/µl) 5µl Cutsmart 10x Buffer 36µl H2O	1µl BglII 1µl XhoI 7µl Vector (145,5ng/µl) 5µl Buffer 3.1 5x Buffer 36µl H2O

for 1h at 37°C.
Purification of fragments was performed using the zymo DNA clean & concentrate kit 5.
Ligation of all three fragments (according to igem texas ligation calculator):

10ng/µl dilution of each fragment.
1µl 10x buffer
1µl T4 ligase
3µl diluted pET28b
2.5µl diluted YebF, 2.5µl diluted GusB (or 5µl water as ctrl)

for 20min at RT.

Transformation with ligated vectors was performed using competent NEB Turbo cells from stock
Unfreezing at 4°C
add 5µl Ligation
10' 4°C
1' 42°C
5' 4°C
add 200µl prewarmed LB
1h 37°C
afterwards, streak 200µl on LB-Kan plates (twice)

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

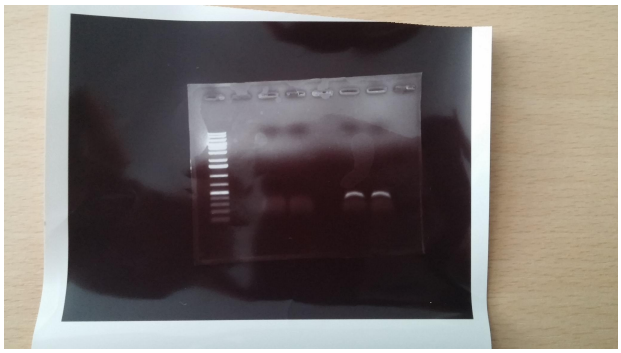
WEDNESDAY, 10/8

PCR for homologue regions of ATG1 in S. cerevisiae.
50µl total:

1. 10µl buffer
2. 2µl dNTPs
3. 2.5µl fwrd
4. 2.5µl rev
5. 0.5µl enzyme
6. 32µl diluted template (9.5ng/µl)

Annealing: 57°C

20160810_141018.jpg



Downstream homologue sequence was amplified.
Unable to amplify Upstream homologue sequence.

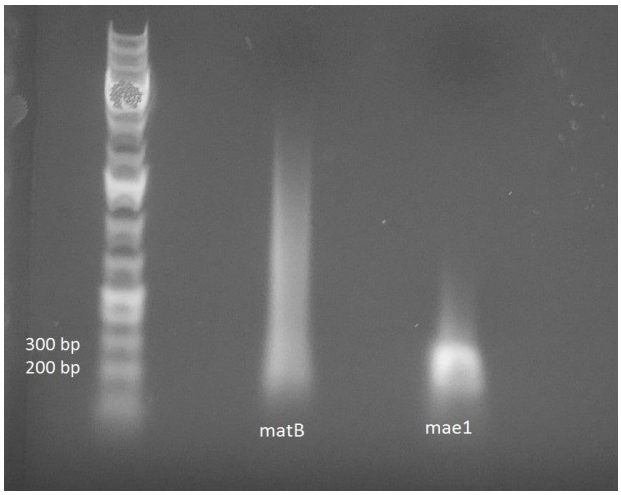
Clean Amplify PCR of the synthesized gens mae1 and matB

50µl total:

1. 10µl buffer
2. 2µl dNTPs
3. 2.5µl fwrd
4. 2.5µl rev
5. 1µl Template
6. 0.5µl Phusion

annealing 57°C.

mae1_matb.jpg



Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

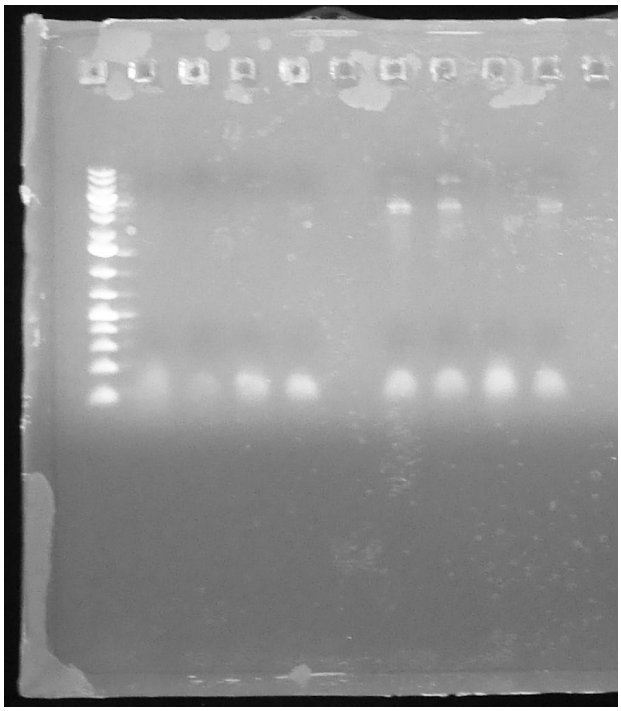
WEDNESDAY, 10/8

Trafo succesful. To verify insert, cPCR was performed (four colonies were picked of each plate):

5µl colony (resuspended in 30µl H2O)
2µl 10mM dNTP
1µl Yebf fw + GusB rev (1:10)
0.5µl Phusion
10µl 5x Phusion Buffer HF
ad 50µl H2O

10' 98°C
30" 95°C|
1' 56°C |x31
2' 72°C |
10' 72°C

cPCR_plate 1&2



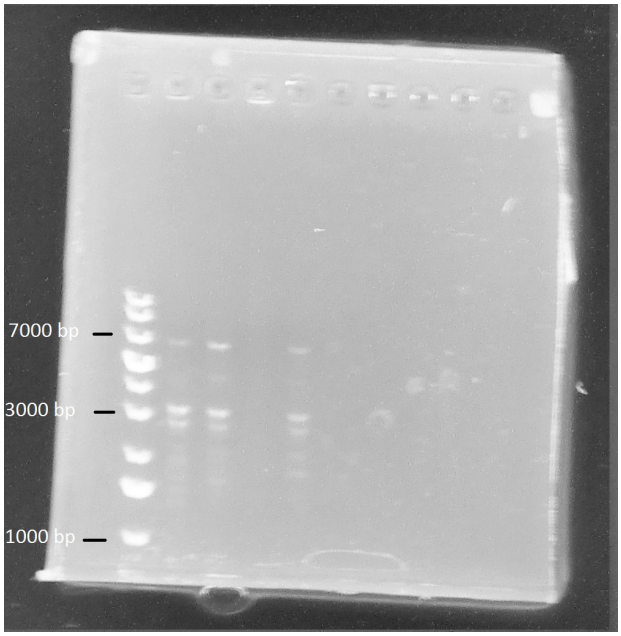
There is no insert in the four colonies of plate 1. Because of the poor seperation of the gen ladder, will repeat the electrophoresis (35 min) with a longer running time for the colonies of plate 2.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

THURSDAY, 11/8

cPCR_plate2.png



Lane 1: Marker; Lane 2-5: cPCR of colonie 1-4

Optained fragments are to big. Might be a problem with the colonie PCR.
Plasmid Prep of colonie 1 and 4 by using zymo prep kit.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

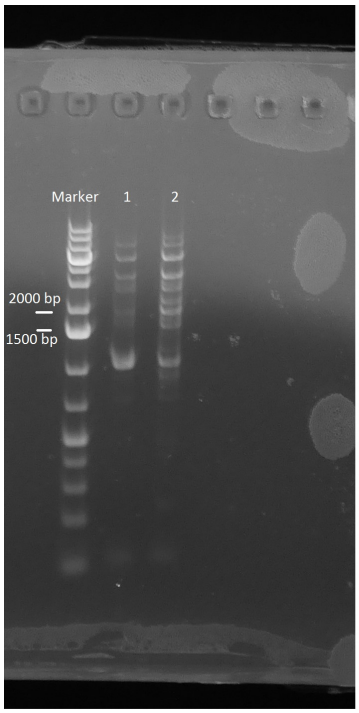
MONDAY, 15/8

To verify insert, PCR was performed of plasmid prep 1 and 4

2µl 10mM dNTP
1µl Yebf fw + GusB rev (1:10)
0.5µl Phusion
10µl 5x Phusion Buffer HF
1µL Tamplate (~10 ng)
ad 50µl H2O

5' 95°C
30" 95°C|
1' 56°C |x31
2' 72°C |
10' 72°C

YebF+GusB_Plasmid1&4_PCR1.png



A lot unspecific fragments. PCR will repated with a reduced annealing time and a higher annealing temprature (see below).

Protein export

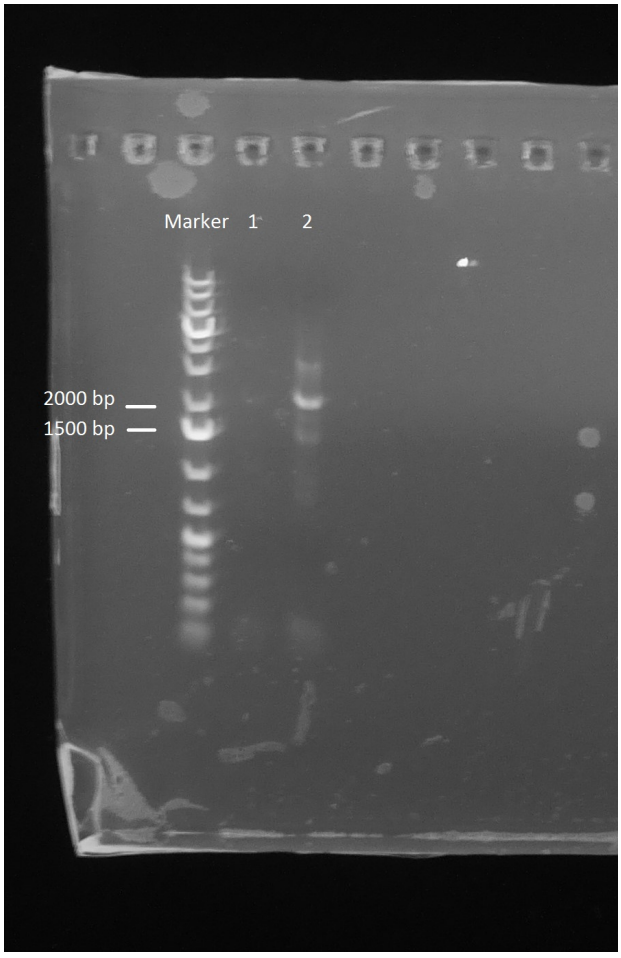
Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

TUESDAY, 16/8

PCR conditions:

5' 95°C
30" 95°C|
30" 60°C |x31
1:10' 72°C |
10' 72°C

YebF+GusB_Plasmid1&4_PCR2.png



Lane 1: Colonie 1; Lane 2: Colonie 4 (see 11.08)

Succesful reduction of unspecific fragments. Colonie 4 contains wanted fragment (~2100 bp).

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

WEDNESDAY, 17/8

Transformation with plasmid of colonie 4 was performed using competent BL21 cells from stock.

Unfreezing at 4°C
add 5µl Ligation
10' 4°C
1' 42°C
5' 4°C
add 200µl prewarmed LB
1h 37°C
afterwards, streak all (~ 200µl) on LB-Kan plate

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

THURSDAY, 18/8

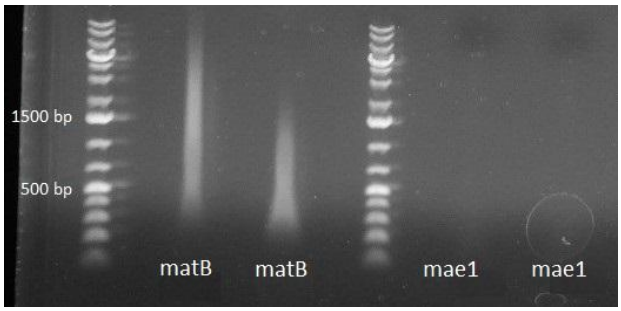
Amplification approach repeated with new primers (mae1 clean fwrd/rev; matB clean fwrd/rev).

total 50µl:

1. 10µl buffer
2. 2µl dNTPs
3. 5µl primer
4. 1µl template
5. 0.5µl phusion polymerase
6. 31.5µl H_2O

Annealing temperature of 63°C.

MatB_Mae1_2.jpeg



Still no amplification.
matB will be ordered again due to unsuccessfully amplification.
mae1 will be amplified by using genomic DNA from *S. pombe* 2698(lov)972 h- strain.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

THURSDAY, 18/8

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

FRIDAY, 19/8

Got Strains with plasmids pJBEI-6410 (Addgene Plasmid: 47049 order: 285639) and pBbA5k-EPL95 (Addgene Plasmid: 45434 order :285639)
Inoculated pJBEI-6410 in 10ml LB+Amp and pBbA5k-EPL95 in LB+Kan

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

SATURDAY, 20/8

Plated pJBEI-6410 on LB+Amp and pBbA5k-EPL95 on LB+Kan.
Prepared Glycerol Stocks of the liquid cultures.
Perfomed a Mini-Prep for both plasmids: pJBEI-6410 (94,3 ng/µl) pBbA5k-EPL95 (126,9 ng/µl). Stored in yellow Box (Production) in -20°C

Transformation:
1: 0,5µl of both cultures in 50µl MG1655 (thawed on ice)
2: 0,75µl pBbA5k-EPL95 and 1µl pJBEI-6410 in 50µl MG1655 (thawed on ice)

25' on ice
45" on 42°C
2' on ice
Added 500µl LB
80' shaking in 37°C Incubator
Plate on prepared Plates

Plates were prepared by adding 200µl a 1:10 dilution of the missing antibiotic (Kan on Amp plates and Amp on Kan plates) on the plates

incubate at 37°C o/n

No colonies

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

MONDAY, 22/8

Panick Topo cloning

4µl of each (mae1 & matB) used for Topo cloning.
Topo cloning reaction transformed into NEB Turbo and streaked out on LB+Amp+X-Gal+ Agar.

- 2x Petrischale
- 2x 50ml Falcon
- 1ml DMF
- 40ml LB-Agar
- 20 ml Amp

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

TUESDAY, 23/8

send pJun in pILS7 (clone9) again for cloning.

Transformation of BHUM2200 (2µl) in NEB Turbo.
30' ice
1' heatshock
30' recovery

plasmid map of pFos is done.
up next:

- [x] Induction of pJun/pFos in BW25113deltaompT
- [x] cloning strategy w/ Nico
- [ ] plasmidmap of pJun

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

WEDNESDAY, 24/8

Amplification of kanMX resistance gene from expression plasmid using pFA6a-KanMX6 as template.

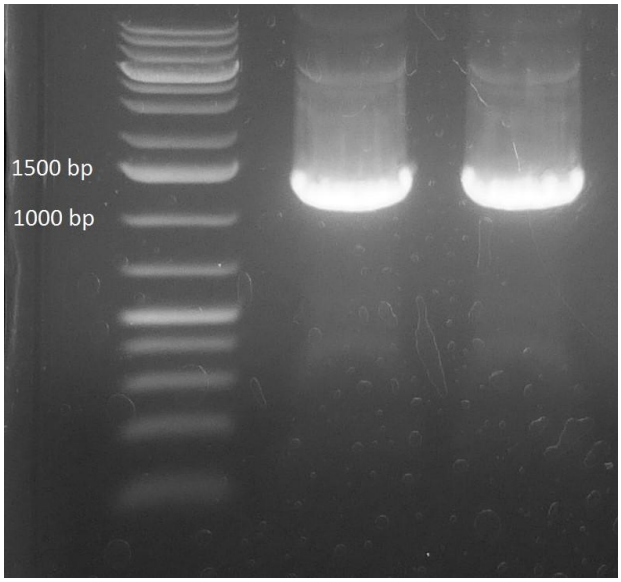
1µl template (~ 1 ng)
2.5µl NK85 primer
2.5µl NK387 primer
1µl dNTPs
10µl 10x phusion high fidelity buffer
32.5µl H2O
0,5µl Phusion polymerase.

and the following PCR setup:

5' 98°C initial denaturation
30" 98°C denaturation |
30" 65°C annealing | x32
50" 72°C elongation |

Estimated band lenghts 1375 bp.

KanMX.jpg



Amplification of the fragment was successful. Gen band was cut out from the gel and cleaned using the Zymoclean™ Gel DNA Recovery Kit.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

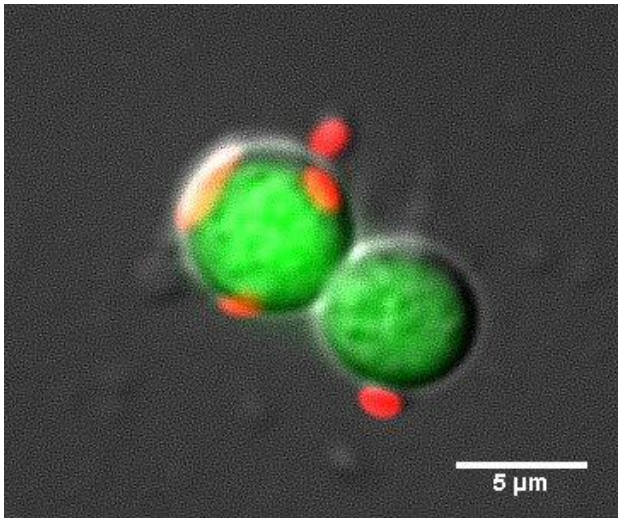
WEDNESDAY, 24/8

PEG protocol with E. coli and a test for regeneration conditions. An E. coli culture with a final dilution of oD 2 was uses.

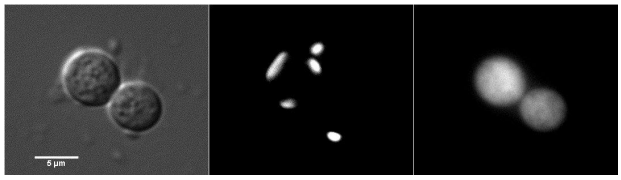
Optimized protocol for Spheroblasts and PEG uptake

Regeneration took place under aerobic conditions in 3ml 1M Sorbitol (in YPD without pH adjustment). Fixation on agar pads was performed, no differences to anaerobic regeneration in 2ml tubes could be seen.

1 merge.jpg



1 montage no label.jpg



Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

THURSDAY, 25/8

The ATG1 KO-Cassette was assambled by Gibson Assembly. Following fragments and volumes were used:

Table1

	A	B	C	D
1	Fragment & Solution	Lenght / bp	Mass / ng	Volumia / µL
2	pUC19 Backbone	2672	46,5	3
3	ATG1 upstream	200	32,4	1
4	ATG1 downstream	200	24,4	1
5	KanMX	1375	258,4	1
6	H2O			4
7	Gibson kit (2X)			10

The mixture was heated to 50°C for 1 h.
Assembled vector was transformend into NEB Turbo using following protocol:

- Unfreezing at 4°C
- add 5 µl assembled vector
- 10' 4°C
- 1' 42°C
- 5' 4°C
- add 200 µl prewarmed LB
- 1h 37°C

afterwards, streak 200 µl on LB-Amp plates.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

FRIDAY, 26/8

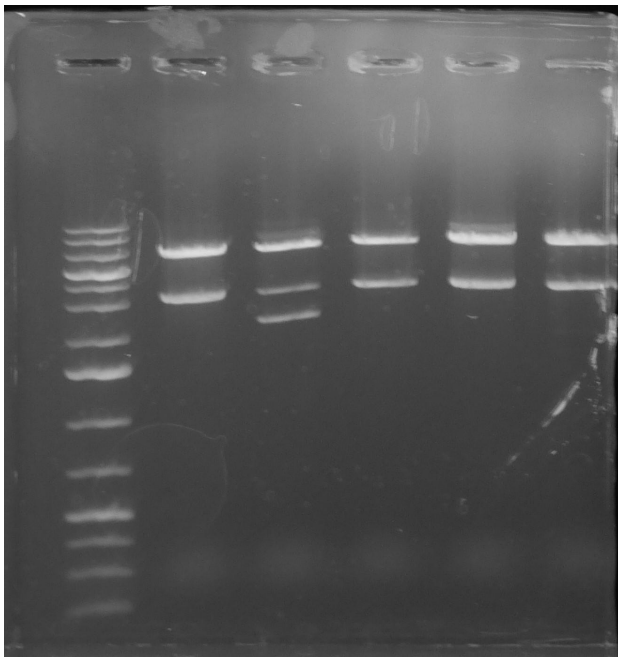
Transformation of pMFM027 (mTq) in NEB Turbo ~200 colonies
Retransformation of pJun in pILS7 (clone 9) no colonies

Analytical Digest for BHUM2200.
Picked 5 Clones. Miniprep.

Table3

	A	B
1	DNA	1µl (~200ng)
2	Buffer (CutSmart) 10x	2µl
3	Enzyme	0.5µl
4	Water	7.5µl

Analytical Digest BHUM2200 - HindIII



Analytical Digest of BHUM2200 w/ HindIII
Expected Bands at 6500bp and 3000bp.
Clones 1, 3, 4, and 5 are correct.
(1%Agarose in TBE Buffer, 1kb Plus Ladder)

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

FRIDAY, 26/8

Transformation was successful.
8 Colonies were picked and incubated in 5 mL LB medium with Amp.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

MONDAY, 29/8

- ☒ Inoculate MG1655 for electrocomp. cells
- ☒ Transformation of pJun-pILS Ligation
- ☒ Inoculate for Induction

Preparative Digest iGEM-Backbones pSB1A3 (AmpR) and pSB1C3 (CamR - shipping BB)

Table4

	A	B	C
1	Plasmid (100ng)	4µl pSB1A3	4µl pSB1C3
2	EcoRI	0.5µl	0.5µl
3	PstI	0.5µl	0.5µl
4	CutSmart Buffer 10x	1µl	1µl
5	H2O	4µl	4µl

incubate at 37°C for 30min

Ligation iGEM-Backbones
Ligation for each Backbone:

Table5

	A	B
1	Plasmid	2µl of Digest
2	T4 Buffer	1µl
3	T4 Ligase	0.5µl
4	H2O	6.5µl

Incubate at room temperature for 1h.

Transformation of pSB1A3(AmpR), pSB1C3(CamR) and pJun-pILS7 Ligation(CamR) in NEB Turbo (2µl each Plasmid).
30' ice
1' heatshock
1h recovery

Cell Aggregation Assay - Quick & Dirty (w/ original plasmids.)
Inoculated 5ml UT5600 pFosß, UT5600 pJunß (CamR), BW25113 pFosß, BW25113 pJunß(CamR), BW25113delta ompT pFosß, BW25113delta ompT pJunß (CamR/KanR). Incubation o/n 37°C.

Cell Aggregation Assay

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

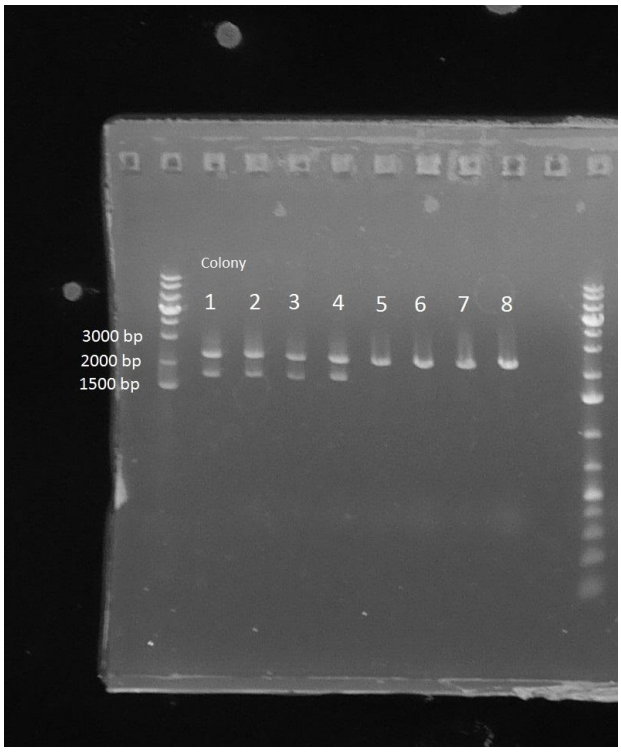
MONDAY, 29/8

Plasmid prep of the 8 colonies by using the Zyppy™ Plasmid Miniprep Kit.
Test Restriction of ATG1 KO-Cassette:

50 µL total
1 µg DNA
5 µL MEB 3.1 buffer (10x)
1 µL PvuI enzyme
1 h at 37°C

Estimated Bands at 2276bp and 1738bp.

Digest of ATG1 KO-Cassette.jpg



The plasmid of the first four colonies were sent to sequenzing.
Sequencing of ATG Ko cassetes. Colony 3 Plasmid was positive.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

MONDAY, 29/8

PEG protocol with E. coli for FACS. Final oD of E. coli = 1.

Optimized protocol for Spheroblasts and PEG uptake

For gates and cell numbers see PDF below. Flow rate was adjusted depending on the cells stability.

16-08-29_iGEM-Batch_Analysis_29082016175859.pdf

No differences between co-culture and endosymbiotic approach could be obtained. Experiment will be repeated using the cell sorter and determining the efficiency of the protocol through micoscropy.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

TUESDAY, 30/8

Inoculated UT5600 pFosß, UT5600 pJunß (CamR), BW25113 pFosß, BW25113 pJunß(CamR), BW25113delta ompT pFosß, BW25113delta ompT pJunß (CamR/KanR)., UT5600, BW25113, BW25113deltaompT.
Grown until OD600 0.5.
No growth.

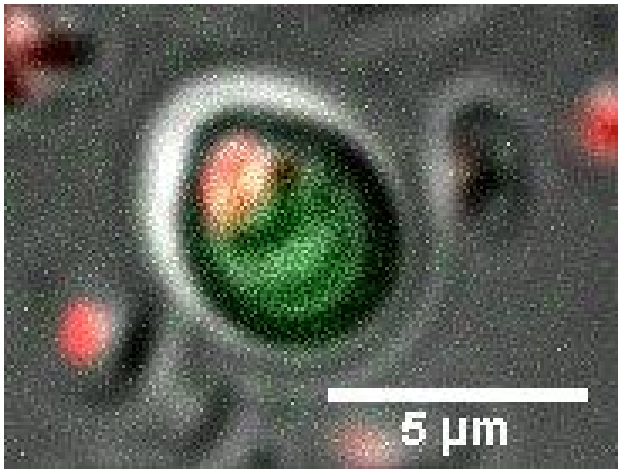
PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

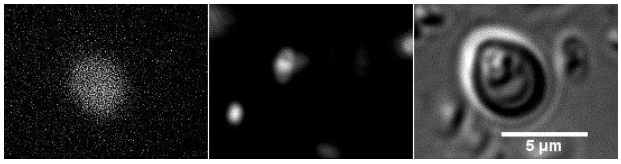
TUESDAY, 30/8

Out of interest, the cells from the FACS experiment (29.08) were used for micoscropy approximately 17h after PEG treatment. Surprisingly, a positive event could be detected. A try to get some z-stacks with this microscope failed miserably.

1 merge.jpg



1 montage.jpg



Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

TUESDAY, 30/8

Inoculate MG1655 in 10 ml LB for electrocompetent cell protocol :
http://openwetware.org/wiki/Richard_Lab:Preparing_electrocompetent_cells

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

WEDNESDAY, 31/8

Inoculated 10ml UT5600 pFosß, UT5600 pJunß (CamR), BW25113 pFosß, BW25113 pJunß(CamR), BW25113delta ompT pFosß, BW25113delta ompT pJunß (CamR/KanR)., UT5600, BW25113, BW25113deltaompT. (9:30)
Induced with 0.5mM IPTG at OD600=0.5-0.6, incubate 3h at 37°C shaking. (10:50)

Mixed equal cell numbers, incubated without shaking. OD600 is measured every twenty minutes. 100µl samples are taken from the top of the liquid.

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

WEDNESDAY, 31/8

Add the 10mL of the overnight culture to each (there are 4) flask containing 200ml of LB medium and incubate at 37°C with vigorous shaking until the OD 600nm is between 0.5 and 1.0. (approximately 3 hours)

Pour the log phase culture into eight 50 mL centrifuge tubes.

Place the tubes on ice for 30 minutes.

Centrifuge for 15 mins at 2000g (3500 RPM) at 4°C.

Remove supernatant and gently resuspend pellets with 30ml ice-cold sterile water.

Centrifuge for 15 mins at 2000g (3500 RPM) at 4°C.

Remove supernatant and gently resuspend pellets in 30ml ice-cold sterile water.

Centrifuge for 15 mins at 2000g (3500 RPM) at 4°C.

Remove supernatant and gently resuspend pellets in 10ml cold 10% glycerol.

Transfer to 15 mL centrifuge tubes and hold on ice for 30 minutes.

Centrifuge for 15 mins at 2000g (3500 RPM) at 4°C.

Remove the supernatant and add 500 µl of 10% glycerol.

Pipet 100µl aliquots into micro-centrifuge tubes (on ice!!!).

Shock freeze cell suspensions using liquid nitrogen and store at -80°C.

Backpacking Cyanos via Leucine Zipper

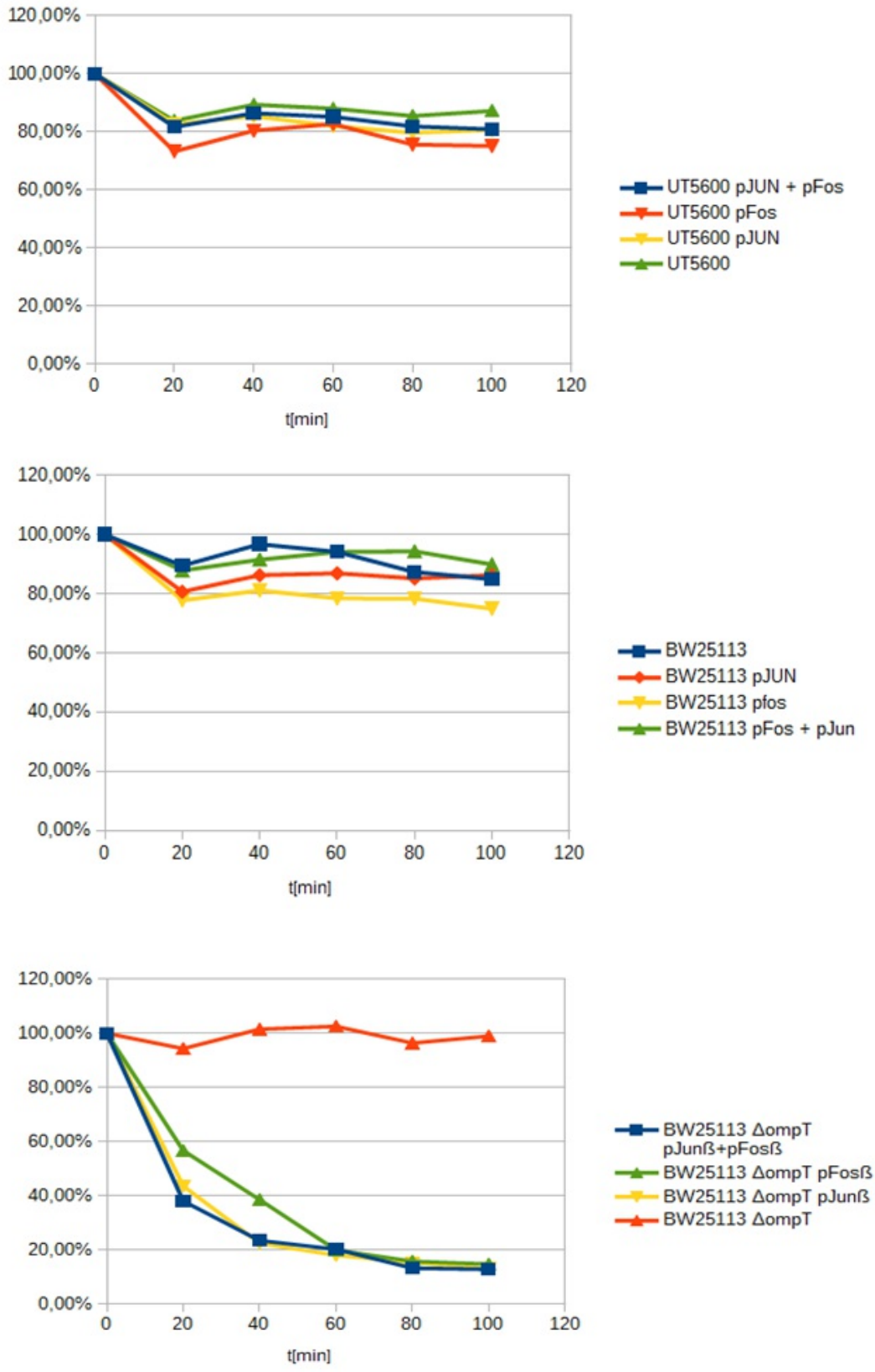
Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

THURSDAY, 1/9

Inoculated 25ml UT5600 pFosß, UT5600 pJunß (CamR), BW25113 pFosß, BW25113 pJunß(CamR), BW25113delta ompT pFosß, BW25113delta ompT pJunß (CamR/KanR), UT5600, BW25113, BW25113deltaompT.
Induced with 0.5mM IPTG at OD600=0.5-0.6, incubate o/n at 37°C shaking.

Mixed equal cell numbers, incubated without shaking. OD600 is measured every twenty minutes. 100µl samples are taken from the top of the liquid.

Cell Aggregation Assay.png



Somehow BW25113 delta ompT Fosß is sedimenting, too. Repeat Transformation.

Table6

	A	B	C	D	E	F	G
1	**Strain/ time**	**0**	**20**	**40**	**60**	**80**	
2	**UT5600**	4,6033333333	3,8566666667	4,1133333333	4,05	3,9333333333	4,01333
3	**UT5600 pJunß**	4,4366666667	3,6666666667	3,7866666667	3,6366666667	3,5333333333	3,57666
4	**UT5600 pFosß**	4,8433333333	3,5466666667	3,8933333333	4,0033333333	3,66	3,63666
5	**UT5600 pJunß + UT5600 pFosß**	4,5566666667	3,72	3,9366666667	3,88	3,73	3,68333
6	**BW25113**	4,4533333333	3,9866666667	4,31	4,1933333333	3,89	3,78333
7	**BW25113 pJunß**	1,5566666667	1,2566666667	1,3433333333	1,3533333333	1,3266666667	1,34333
8	**BW25113 pFosß**	4,01	3,12	3,2533333333	3,1466666667	3,1433333333	3,00666
9	**BW25113 pJunß + BW25113 pFosß**	1,6533333333	1,4533333333	1,5133333333	1,5566666667	1,56	1,48666
10	**BW25113deltaompT**	0,65	0,6133333333	0,66	0,6666666667	0,6266666667	0,64333
11	**BW25113deltaompT pJunß**	3,1566666667	1,3733333333	0,7066666667	0,5666666667	0,48	0,41
12	**BW25113deltaompT pFosß**	2,9533333333	1,6733333333	1,1366666667	0,5866666667	0,4633333333	0,43333
13	**BW25113deltaompT pJunß + pFosß**	3,01	1,1433333333	0,7033333333	0,6066666667	0,3966666667	0,38333

OD600 of each construct. OD's are triplicates.

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

FRIDAY, 2/9

Tranformation of electrocompetent cells with pJBEI-6410 and pBbA5k-EPL95

Procedure

1. Chill the # electroporation cuvettes by floating them in an ice bath.
2. Remove # vials containing 100µl electro-competent cells from the -80°C freezer and thaw them with the iced cuvettes.
3. Prepare # micro-centrifuge tubes containing 900µl SOB media.
4. Turn on electroporator and set voltage to 1.5 kV (1mm cuvettes).
5. Add add 5µL of ligated DNA sample to 100µl thawed electrocompetent cells on ice. Swirl tip around gently in cells to mix DNA and cells.
6. Place cells back on ice to ensure they remain cold.
7. Pippette 100µL of cell-DNA mixture to cuvette.
8. Wipe off excess moisture from outside of cuvette.
9. Place cuvette in chamber of electroporator.
10. Pulse the cells by pressing button on electroporator twice.
11. Quickly use a pipette to remove the electroporated cell suspension from the cuvette and add it to a tubes containing 1ml SOB.
12. Let cells recover at room temperature for 30-60 minutes.
13. Plate 100µl of eletroporated cells onto prewarmed LB-agar plate supplemented with appropriate antibiotic. Incubate plate overnight at 37°C.

Notes

- When choosing the # of vials and cuvettes, be sure to include 2 vials for two negative controls (one with no DNA added, and another with only cut vector added).

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

MONDAY, 5/9

No colonies

Transformation of electrocompetent MG1655 (on Amp Kan Plates)
Parallel Transformation of chemocompetent NEB Turbo with pJBEI-6410 and pBbA5k-EPL95 (0,75µl pBbA5k-EPL95 and 1µl pJBEI-6410)

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

TUESDAY, 6/9

Ligation of pJun/pILS7
1µl vector (pILS7, 2,935bp, 21.6ng/µl)
6µl Insert (pJun, 1,500bp, 8.4ng/µl)
2µl buffer
1µl ligase
10µl H2O

incubate at room temperature for 1h.

Transformation of 2µl in NEB Turbo.
30' ice
1' heat shock at 42°C
1h recovery. plated on LB-Cam.

Inoculation of BW25113deltaompT for competent cells.
Inoculation of pJun and pFos for Mini Prep

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

TUESDAY, 6/9

Colonies found on NEB Plates (not on ctrl)
No colonies on MG 1655 plates

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

WEDNESDAY, 7/9

pJun did not grow, pFos grew. pFos was spun down, froze pellet.

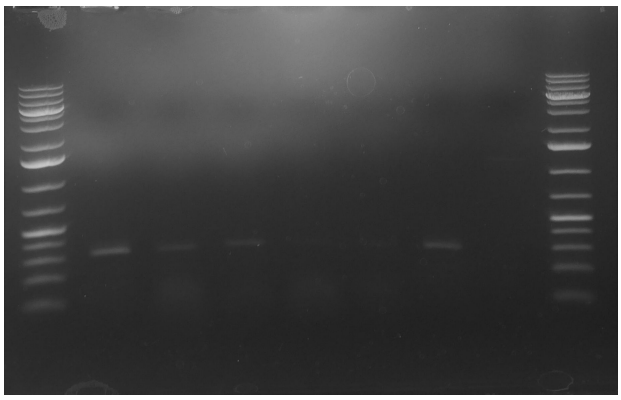
Tranformation pJun/pILS7 in NEB Turbo 6 colonies.
colony PCR of clones.

Table7

	A	B	C	D	E	F
1	**Number of reactions**	**8**	**Master Mix**	**PCR Program**		
2						
3	**Dream Taq (2x)**	10	80	98°C	10'	
4	**Primer VR**	1	8	95°C	30"	|
5	**Primer VF2**	1	8	55°C	45"	| x32
6	**DMSO**	1	8	72°C	90"	|
7	**H2O**	7	56	72°C	5'	

after PCR probes are run on a 1% Agarose in TBE.

colony PCR Ligation pJun pILS7.JPG

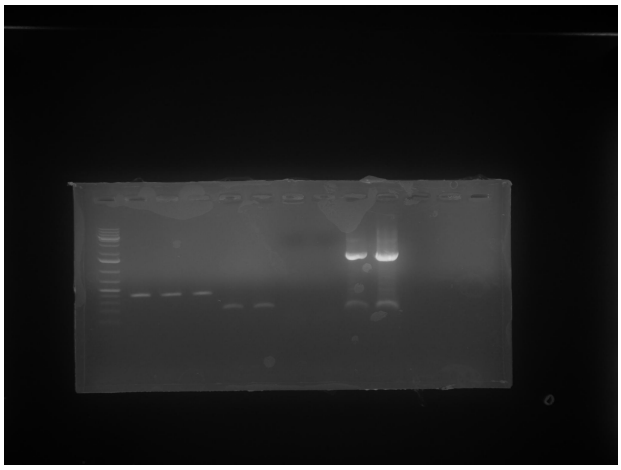


Lane 1: 1kb Plus DNA Ladder, 6µl
Lane 2: clone 1
Lane 3: clone 2
Lane 4: clone 3
Lane 5: clone 4
Lane 6: clone 5
Lane 7: clone 6
Lane 8: pILS7
Lane 9: Marker, 6µl

Expected bands at 2,000bp, bands at 300bp are religated vector. For pILS7 without pJun 1,200bp are expected. only a very weak band.

☒ competent cells of BW25113deltaompT
Transformation of ligation pJun pILS7 is repeated. 2 clones. negative.

P1000253.JPG



Lanes 7 & 8 are the tested clones.

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

FRIDAY, 9/9

Amplification of panD and ppc gene from synthesized DNA product.

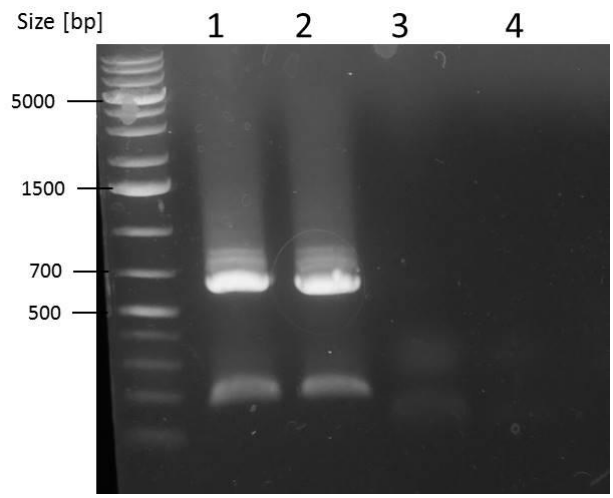
1µl template (~ 1 ng)
2.5µl PanD safe fwrd primer
2.5µl PanD safe rev primer
1µl dNTPs
10µl 10x phusion high fidelity buffer
32.5µl H2O
0.5µl Phusion polymerase.

and the following PCR setup:

1' 98°C initial denaturation
30" 98°C denaturation |
30" 61°C (panD) 58°C (ppc) annealing | x32
20" (panD) 1.30' (ppc) 72°C elongation |
7' 72°C terminal extension

Estimated band lenghts 599 bp for panD and 2698 bp for ppc.

PCR_PanD and PpC.jpg



Amplification of the synthesized panD product was successful as seen on lane one and two. Gen band was cut out from the gel and cleaned using the Zymoclean™ Gel DNA Recovery Kit. There was no band seen for ppc (lane three and four). Will repeat it with lower annealing temprature.

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

FRIDAY, 9/9

Streaking out of NEB Turbo + pJBEI-6410 pBbA5k-EPL95 and preparation of Glycerol-Stock

Preparation of SOB-Medium for electrocompetent MG1655 (2L)
Per liter:

- 5 g yeast extract
- 20 g tryptone
- 0.584 g NaCl
- 0.186 g KCl
- 2.4 g $MgSO_4$

Colonies found for NEB and no colonies for MG1655

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

FRIDAY, 9/9

New attempt using a FliC-POI construct. As reference, mRFP from the igem registry is used (BBa_I13521 in backbone with canamycine resistance). Transformation into competent NEB Turbo was done after resuspending the DNA in 10µl ddH2O and storing it on -20°C.
Thawing of competent cells at 4°C
add 1µl of resuspended DNA
10' 4°C
1' 42°C
5' 4°C
add 200µl prewarmed LB
1h 37°C
afterwards, streak 100µl on LB-Cam plate

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

SATURDAY, 10/9

Induction of transformed NEB culture with 500µM (10µl of stock), 100µM (2µl) and 25µM (0,5µl) at OD600 0,54.

Inoculated MG1655 for electrocompetent cells.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

SATURDAY, 10/9

Trafo was not succesful. New competent NEB Turbos were prepared using the given protocol:

E. coli Calcium Chloride competent cell protocol

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

SUNDAY, 11/9

First try PCR assembly of P450:

Table1

	A	B	C	D	E	F	G
1	Water	Primer fwd	Primer rev	5x Phusion buffer	dNTPs	Templates	Phusion
2	11,3µl	1µl	1µl	4µl	1µl	0,75 + 0,75µl	0,2 µl
3	10,8µl	1µl	1µl	4µl	1µl	1 + 1µl	0,2 µl

Table2

	A	B	C
1	1 Cycle	98°C	2'
2	35 Cycles	98°C	30"
3		58°C	1'
4		72°C	1'20"
5	1 Cycle	72°C	5'
6	1 Cycle	4°C	storage

Inoculated NEB Turbo + pJBEI-6410 + pBbA5k-EPL95 in Kan+Amp Medium

Dependencies

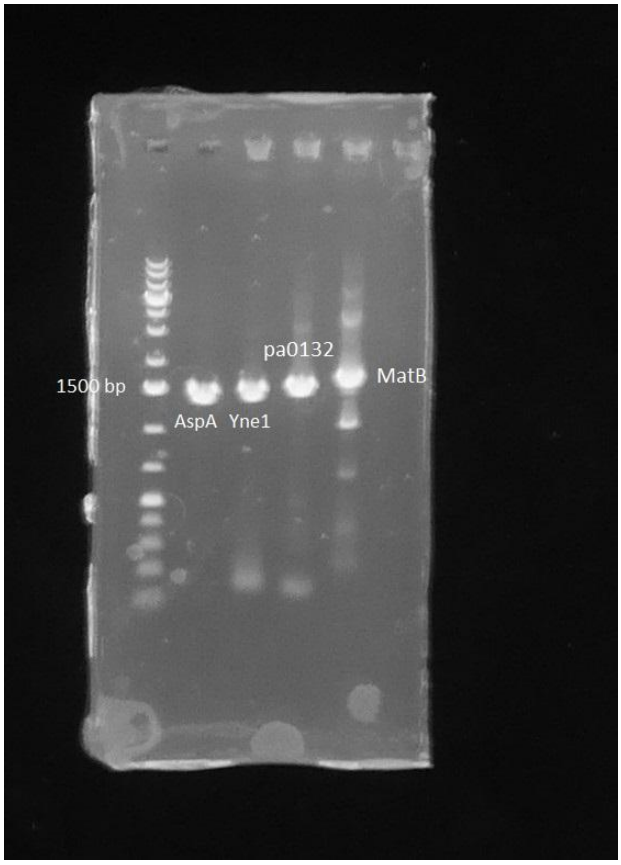
Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

MONDAY, 12/9

Amplification of aspA and yne1 gene from genomic e. coli DNA, pa0132 and matB from synthesized DNA product.
Using the same protocol as on 08/24, but with an annealing temprature of 58°C.

Estimated band lenghts 1437 bp (aspA), 1466 bp (yne1), 1488 bp (p0132) and 1545 (matB)

PCR_AspA, Yne1, pa0132, MatB.JPG



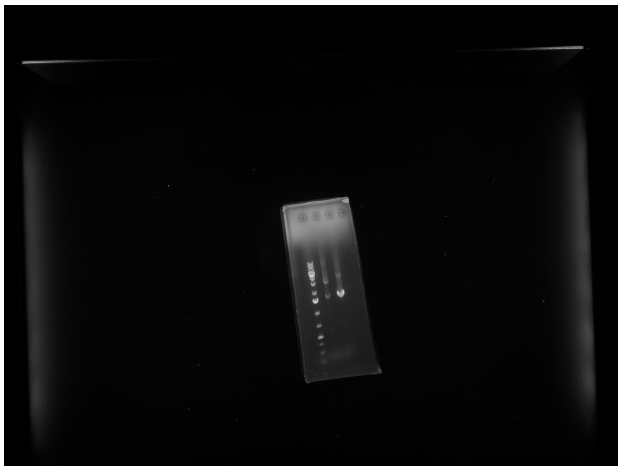
All four PCR were successful. aspA, yne1 and pa0132 were purified using the zymo DNA clean & concentrate kit.
matB was again seperated by gel electrophoresis and the gen band was cut out and cleaned using the Zymoclean™ Gel DNA Recovery Kit.

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

MONDAY, 12/9

P1000262.JPG



PCR assembly gel shows events at 1500bp and 3000 bp (Generuler 1kb plus).
(Marker; 1; 2)

Inoculated 50ml of Limonene culture

induced with IPTG (25, 100, 500 and 500 µM for 37°C) at OD600 0,8

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

MONDAY, 12/9

New transformation using the same procedure as before (9.9.).

Production

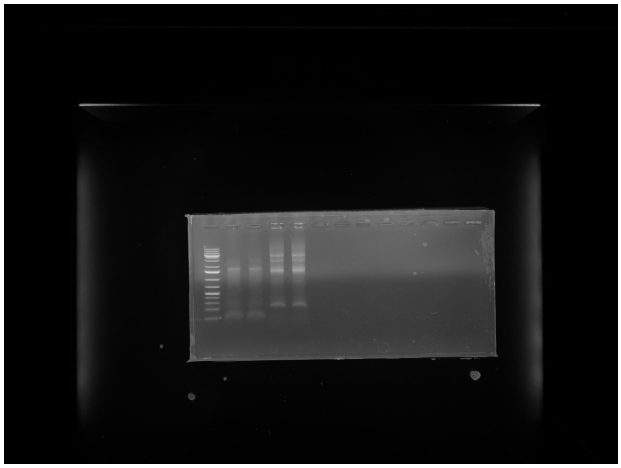
Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

TUESDAY, 13/9

Lysis of induced cells via sonication (50% 2x30' with 30' pause)

PCR assembly with GcABC-G1 and P450 Templates

1709.JPG



First two lanes: P450 assembly; slight Bands visible around 3000bp (Generuler 1kb plus).
Other two lanes: GcABC-G1 assembly; slight bands visible around 3000bp and above 4000 bp (those were cut out)

Gel exrtaction of bands that were cut out (25,7ng/µl)

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

TUESDAY, 13/9

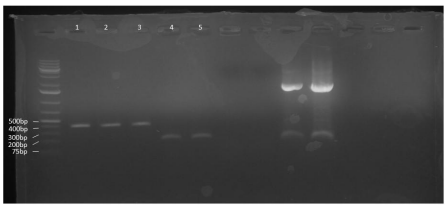
1 positive colony from transformation could be detected and was incubated in 15ml LB + Cam.

To amplify the fliC signal sequence and yebF with a new reverse primer (with His-tag), following PCRs were performed:
FliC
5µl colony (resuspended in 30µl H2O)
1µl 10mM dNTP
1µl FliC fw + FliC rev Gib (1:10)
1µl Phusion
10µl 5x Phusion Buffer HF
ad 50µl H2O

YebF
5µl colony (resuspended in 30µl H2O)
1µl 10mM dNTP
1µl YebF fw + YebF His rev (1:10)
1µl Phusion
10µl 5x Phusion Buffer HF
ad 50µl H2O

And both on these conditions
5' 95°C
30" 95°C |
30" 54°C |x30
1" 72°C |
10' 72°C

13.09.JPG



lanes 1-3 represent YebF (~400bp with His-tag), lanes 4-5 represent FliC (193bp). Marker: GeneRuler kb plus

Both PCRs were successful and samples were stored at 4°C for following purification.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

WEDNESDAY, 14/9

Transformation of 5µl Ligation pJun pILS7 in NEB Turbo.

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

WEDNESDAY, 14/9

Inoculation of new culture of NEB + pJBEI-6410 + pBbA5k-EPL95 in 10 ml LB+Kan+Amp

Performing a Bradford assay of the lysed cells to equalize the samples (8µg/µl).

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

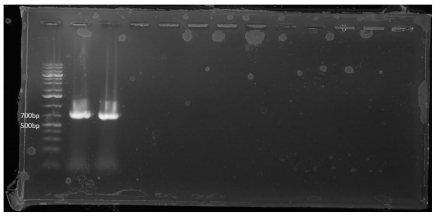
WEDNESDAY, 14/9

Purification of YebF and FliC samples was performed using the Zymo Cleanup kit. Samples were stored at -20°C afterwards.

To verify the transformation with mRFP from the registry, the plasmids from inoculated Turbos were prepped (Zymo Plasmid prep kit) and used as a template for PCR with corresponding primers:
10µl DreamTaq Mastermix (2x)
1µl DMSO
1µl mRFP fw Gib + mRFP rev (1:10)
1µl Template (Plasmid, which has been diluted to 1ng/µl before)
ad 20µl H2O

5' 95°C
30" 95°C |
30" 57°C |x30
2" 68°C |
5' 68°C

14.09.JPG



Exprected lanes at ~700bp (size mRFP: 683bp). Marker: GeneRuler kb plus

For transformation of NEB Turbos with YebF in pET28b the following digestion has been perforemed:
20µl concentrated yebF (38.3ng/µl)
5µl Cutsmart (5x)
1µl NcoI
1µl XhoI
ad 50µl H2O

for 1h at 37°C. Afterwards heat inactivation on 80°C for 20' and purification using the Zymo Clean&concentrator kit.
Ligation with the plasmid (from stock) with volumes calculated with igem ligation calculator:
1µl T4 Ligase Buffer
1µl T4 Ligase
0.5µl H2O
5µl pET28b (77.8ng/µl)
2.5µl yebF (23.5ng/µl)
for 10' at RT.

Then, transformation of competent NEB Turbo using the standard procedure:
Unfreezing at 4°C

add 5µl Ligation
10' 4°C
1' 42°C
5' 4°C
add 200µl prewarmed LB
1h 37°C
afterwards, streak ~150µl on LB-Kan plate and let incubate over night.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

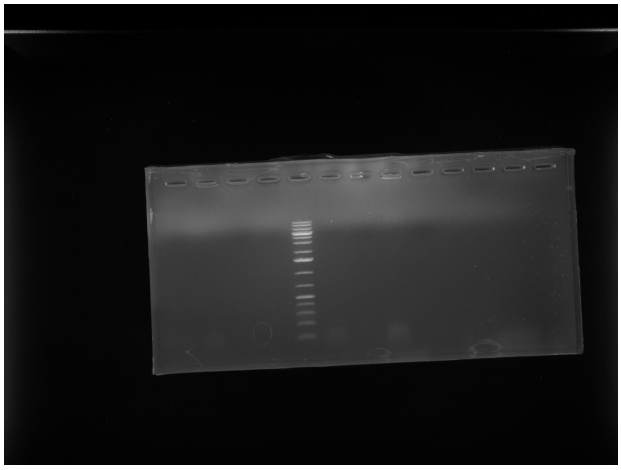
THURSDAY, 15/9

Transformation showed ~20 clones
colony PCR of 8 clones.

Table8

	A	B	C	D	E	F
1	**Number of reactions**	**8**	**Master Mix**	**PCR Program**		
2						
3	**Dream Taq (2x)**	10	80	98°C	10'	
4	**Primer VR**	1	8	95°C	30"	|
5	**Primer VF2**	1	8	55°C	45"	| x32
6	**DMSO**	1	8	72°C	90"	|
7	**H2O**	7	56	72°C	5'	

P1000258.JPG



lanes 1-4: clones 1-4
lane 5: 6µl marker
lane 6-9: clones 5-8

somehow PCR did not work. same protocol as before. i doubt that its just false positives. will talk to basti about it. also maybe the dream taq aliquot was off?

Production

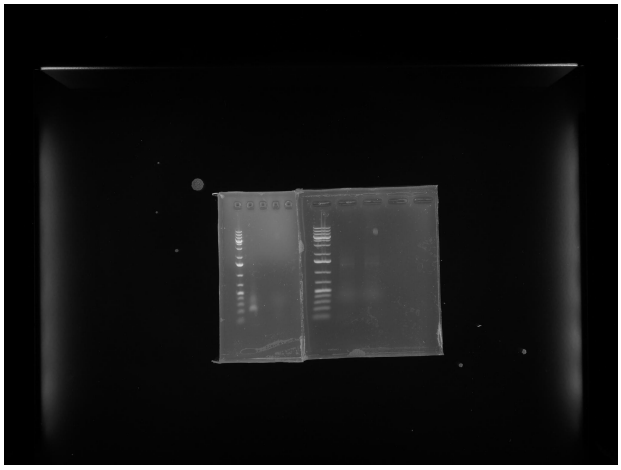
Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

THURSDAY, 15/9

Induction of cells at OD600 0,74 (50ml) with IPTG (25µM, 100µM, 500µM)

PCR assembly of P450 (big gel)and PCR of extracted GcABC-G1 (small gel):

2009.JPG



No distinct bands visible (Generuler 1kb plus)..

Inoculation of new culture of NEB + pJBEI-6410 + pBbA5k-EPL95 in 10 ml LB+Kan+Amp

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

THURSDAY, 15/9

Positive clones detected, verification via cPCR. Turned out this was useless, because YebF is in the genome of NEB Turbo, too...

10µl DreamTaq Mastermix (2x)
1µl DMSO
1µl YebF fw + YebF Gib rev (1:10)
5µl colony (diluted in 30µl H2O)
ad 20µl H2O

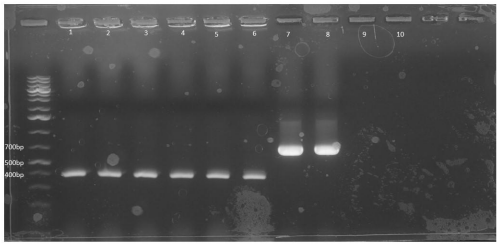
5' 95°C
30" 95°C |
30" 57°C |x30
2" 68°C |
5' 68°C

mRFP cultures were harvested and the plasmid prepped according to the Macherey&Nagel Plasmid kit.
Yields: mRFP1 - 229.1ng/µl; mRFP2 - 240.4ng/µl. Verification through PCR.

10µl DreamTaq Mastermix (2x)
1µl DMSO
1µl mRFP fw Gib + mRFP rev (1:10)
1µl Template (Plasmid, which has been diluted to 1ng/µl before)
ad 20µl H2O

5' 95°C
30" 95°C |
30" 57°C |x30
2" 68°C |
5' 68°C

15.09.JPG



lanes 1-6 are YebF (~400bp) from cPCR. No verification possible due to occuring YebF in the genome, too. Lanes 7+8 are mRFP (~700bp) with plasmids as templates. Lane 7 - mRFP1; lane 8 - mRFP2. Marker: Generuler kb plus

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

FRIDAY, 16/9

Lysis of cells via sonication (50%, 2x 30', Puase: 30')

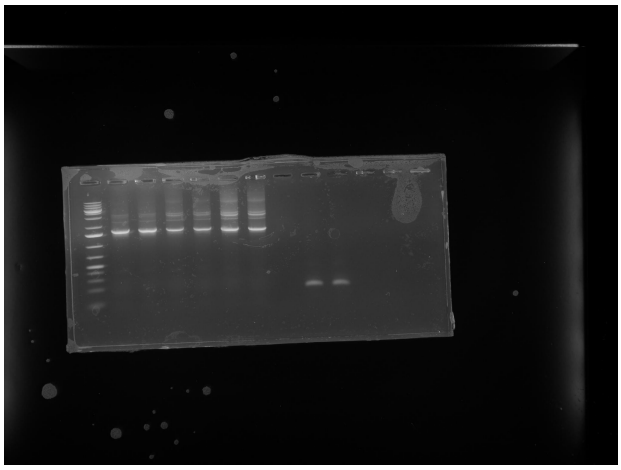
Bradford: 19µg/µl

Induction of cells at OD600 0,6 (50ml) with IPTG (25µM, 100µM, 500µM)

PCR-assembly of P450: lanes 1+2: 0,5+0,5µl Template (60bp overhang)
lanes 2+3: 0,75+0,75µl Template(60bp overhang)
lanes 4+6: 1µl+1µl Template (60bp overhang)

strong bands visible at 3000bp (Generuler 1kb plus). Bands were cut out, proceeded with a pooled gel extraction: 57,5ng/µl

210916 P450.JPG



Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

FRIDAY, 16/9

mRFP was amplified for the later use for Gibson assembly with FliC.

1µl Template (Plasmids diluted to 1ng/µl)
1µl 10mM dNTP
1µl mRFP fw Gib + mRFP rev (1:10)
1µl Phusion
10µl 5x Phusion Buffer HF
1µl DMSO
ad 50µl H2O

2' 98°C
30" 98°C |
30" 56°C |x30
1'20" 72°C |
5' 72°C

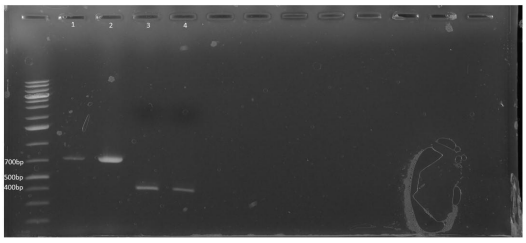
Results see gel picture

pET28b with YebF was prepped using the Macherey&Nagel plasmid kit (Yields: YebF2 - 716.4ng/µl; YebF3 - 636.9ng/µl), verification followed doing a normal PCR

10µl DreamTaq Mastermix (2x)
1µl DMSO
1µl YebF fw + YebF rev Gib (1:10)
1µl Template (YebF2 and YebF3, which have been diluted to 1ng/µl before)
ad 20µl H2O

2' 95°C
30" 95°C |
30" 58°C |x30
1' 68°C |
5' 68°C

16.09.JPG



lanes 1+2 mRFP as expected at around 700bp. Lanes 3+4 YebF with bands at expected 400bp. Marker: GeneRuler kb plus

Amplified mRFP was purified using Zymo DNA concentration kit (Yields: mRFP1 - 13.9ng/µl; mRFP2- 56.3ng/µl) and samples stored at -20°C.
Since results for YebF insertion into pET28b was succesful, the plasmids were used for transformation into BL21 competent cells (from lab stock).
Unfreezing at 4°C
add DNA (100ng)
10' 4°C
1' 42°C
5' 4°C
add 200µl prewarmed LB
1h 37°C
afterwards, streak ~150µl on LB-Kan plate and let incubate over night.

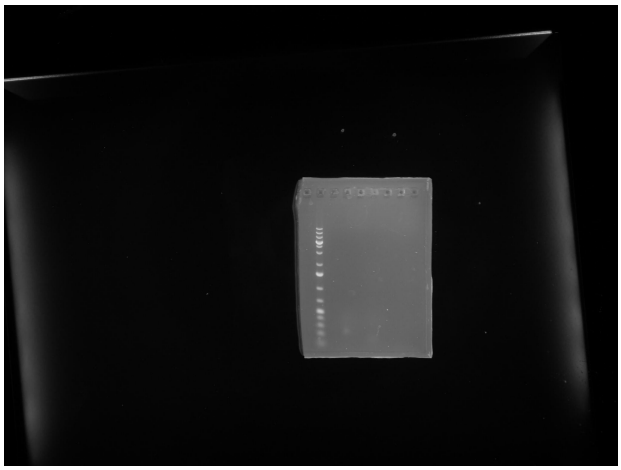
Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

SATURDAY, 17/9

Phusion-PCR of P450 and GcABC-G1: no bands visible (Generuler 1kb plus).

P1000296.JPG



Lysis of cells 24h after induction via sonication(50%, 2x30', 30' Pause)

Bradford:

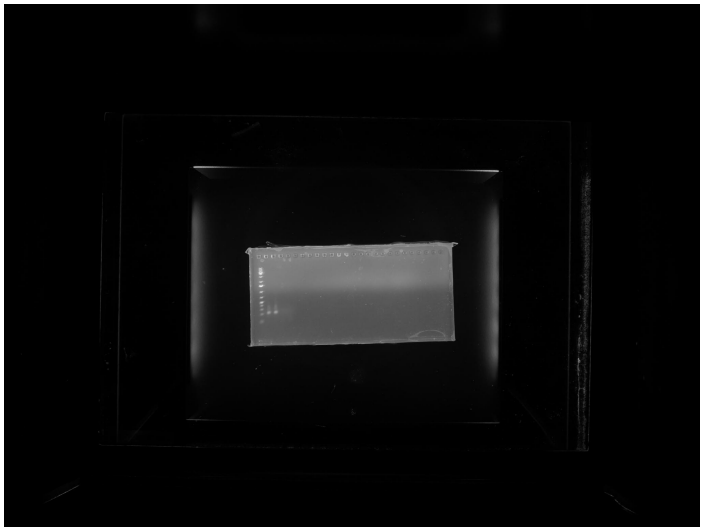
Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

SUNDAY, 18/9

PCR of P450, GcABC-G1 and Hygromycin cassette from pfaa6-hph nt plasmid: no bands visible except unspecific ones in case of GcABC-G1 (1+2)

230916 GcABC-G1 1 2 P450 Hyg.JPG



Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

MONDAY, 19/9

Gibson Assembly of Gibson 1 (panD, aspA, pa0132, pUC19) following with a transformation and streak on LB-Amp plates.
The method was the same as on 08/25.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

MONDAY, 19/9

3% agarose in 1M Sorbitol (1.2g in 40ml 1M Sorb).
Inoculation of YMFM in 15ml YPD for test of agarose pads.

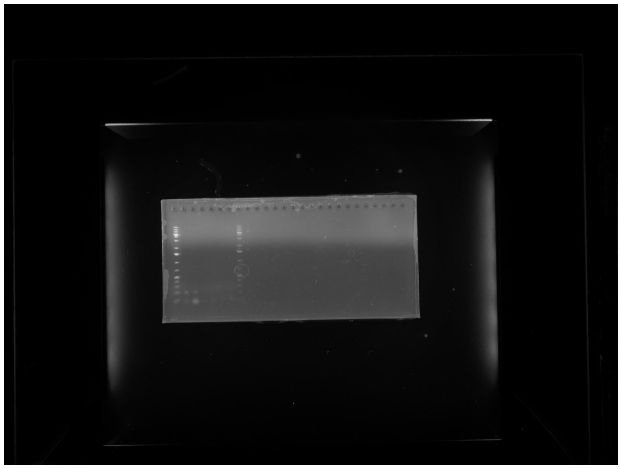
Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

MONDAY, 19/9

PCR of GcABC-G1 (Template 1:20), P450 (Template: 1:50) and Hygromycin cassette: no bands visible (Generuler 1kb plus).

270916 GcABC-G1 1 2 P450 3 4 Hyg 5 6.JPG



Preparation of EZ-rich medium:

Table3

	A	B	C
1	Part #	Description	Amount
2	M2101	10X MOPS Mixture	100 mL
3	M2102	0.132 M K2HP04	10 mL
4	M2103	10X ACGU	100 mL
5	M2104	5X Supplement EZ	200 mL
6	W0225*	Sterile H20*	585 mL
7	G0520	20% Glucose	5 mL
8		Total	1000 mL

inoculation of NEB Turbo + pJBEI-6410 + pBbA5k-EPL95 in 10ml LB+Kan+Amp

Lysis of cells 24h after induction via sonication(50%, 2x30', 30' Pause)

Preparation of Bradford.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

MONDAY, 19/9

Positive clones for transformation of BL21 with pET28b with YebF could be detected. Inoculation of one each plate in 25ml LB-Kan for induction with IPTG.
Induktion with 30µM IPTG was performed at an oD of 0.66 respectively 0.86. At different timepoints (before induction, 1h after induction, 2h after induction) 1ml culture was obtained and centrifuged. Supernatant (80µl) as well as pellet (resuspended in 80µl H_2O) were mixed with 20µl SDS-Buffer and the samples stored at -20°C.

Backpacking Cyanos via Leucine Zipper

Project: iGEM 2016
Authors: Maria Lindner
Dates: 2016-07-15 to 2016-09-20

TUESDAY, 20/9

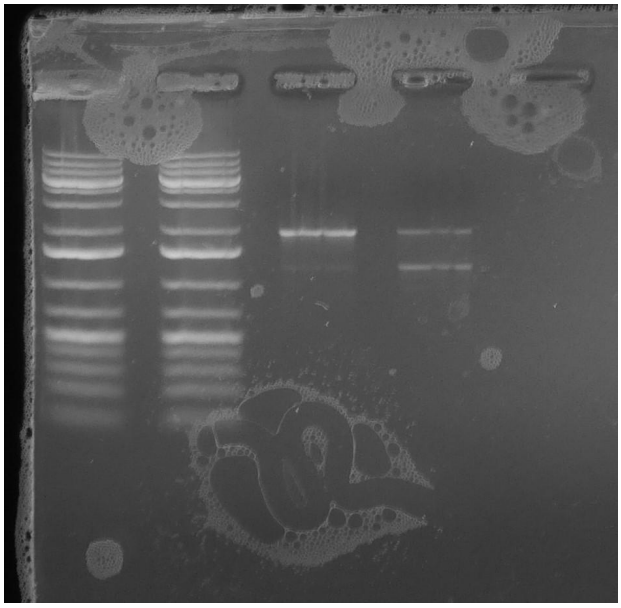
aliquot was o.k. - did pcr again with basti. improved program. just tried two clones.

Table9

	A	B	C	D	E	F
1	**Number of reactions**	**2**	**Master Mix**	**PCR Program**		
2						
3	**Dream Taq (2x)**	10	20	98°C	5'	
4	**Primer VR**	1	2	95°C	30"	|
5	**Primer VF2**	1	2	52°C	45"	| x32
6	**DMSO**	1	2	68°C	90"	|
7	**H2O**	7	14	68°C	5'	

changes marked in orange. according to thermo fisher the annealing temp for the primers should be 52°C, igem gives 55°C.

PCR pJunpILS Ligation in NEB Turbo clones 16 and 17.jpg



Lane 1: Marker (was broken.)
Lane 2: Marker (6µl)
Lane 3: clone 16
Lane 4: clone 17

PCR product of correct clones should be ~ 2000bp. slight band at 1.500bp is seen in clone 16, will mini prep this and check on gel before sequencing.

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

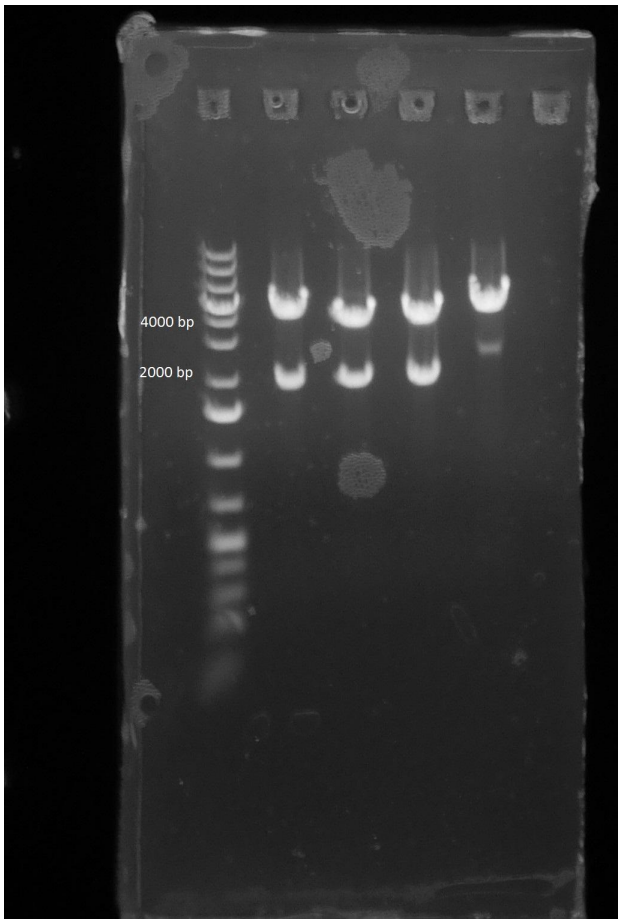
TUESDAY, 20/9

Four colonies were picked and incubated in 5 mL LB medium with Amp.
Plasmid prep of the colonies.
Test Restriction of Gibson1-Cassette:

50 µL total
1 µg DNA
5 µL CutSmart buffer (10x)
1 µL KpnI enzyme
1 h at 37°C

Estimated bands at 3818 bp and 1911 bp.

Gibson1_test digestion_Kpn1.jpg



Colonies 1-3 positive. Continuing with colony 2.

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

TUESDAY, 20/9

SDS-PAGES performed with the lysed cell samples.

gels have been running at 100V, 50mA thorugh stacking and 180V, 50mA through resolving gel til the running band reached the bottom of the gel.

2 gels were stained with coomassie staining for 30 minutes and deastained with water over night.

2 gels were blotted on nitrocellulose membranes via semidry blotting procedure. (205mA, 15V, 2h)

membranes were blocked with 5% milk solution over night at RT.

inoculation of NEB Turbo + pJBEI-6410 + pBbA5k-EPL95 in 10ml LB+Kan+Amp

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

TUESDAY, 20/9

After approximately 17h of induction, purification using Ni-beads was done (see 27.5 for procedure). Cell lysis was performed via sonification instead of microfluidizer (4x 30s on 50% with 30s breaks inbetween).
All of the samples were run over a SDS-page:

PICTURE SOON

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

WEDNESDAY, 21/9

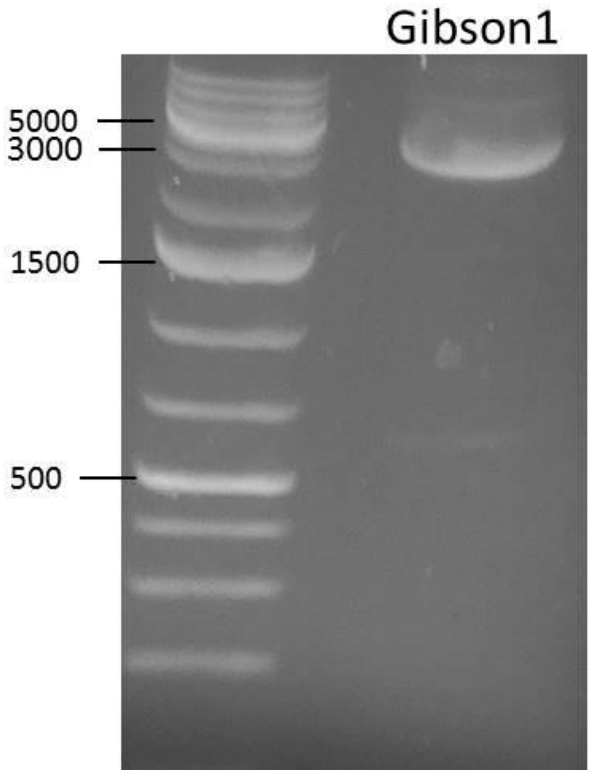
For the final Gibson assambly of the operon a genfragment from Gibson 1 was amplified by PCR including the gens panD, pa0132 and aspA:

1µl template (~ 1 ng)
- 2.5µl panD fwrd primer
- 2.5µl pa0132 rev primer
- 2µl dNTPs
- 10µl 10x phusion high fidelity buffer
- 31.5µl H2O
- 0.5µl Phusion polymerase.

and the following PCR setup:
- 1' 98°C initial denaturation
- 30" 98°C denaturation |
- 30" 58°C annealing | x32
- 2.5' 72°C elongation |
- 7' 72°C terminal extension

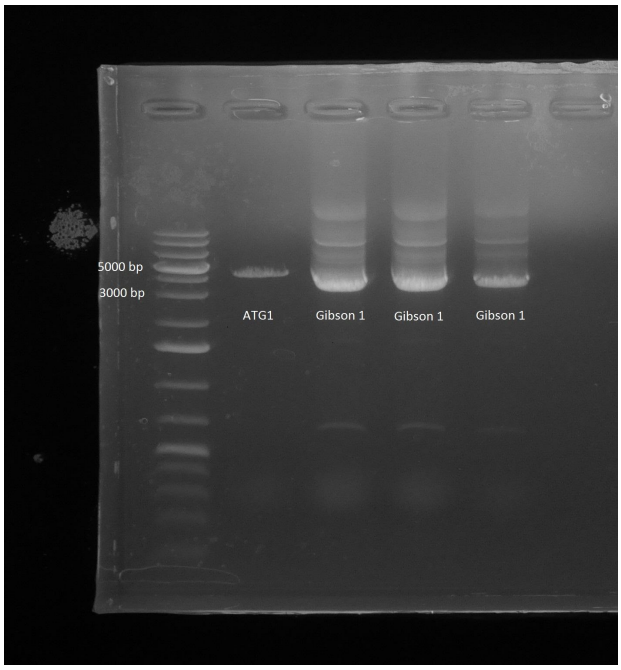
Estimated band lenghts 5421 bp.

Gibson_1_PCR.JPG



Due to low yield the amplification will be repeated.
Additionaly the vector length of the ATG1 KO Cassette (3890 bp) was verified using a restriction digestion with SapI.

ATG1_KO_Digestion +Gibson_1_Amplification.jpg



Gen band of Gibson1 was cut out from the gel and cleaned using the Zymoclean™ Gel DNA Recovery Kit.

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

WEDNESDAY, 21/9

PEG protocol with E. coli for micoscropy with confocal laser scan microscopy (it makes nice z-stacks).

Z-stacks showed no uptake, repitition will follow.

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

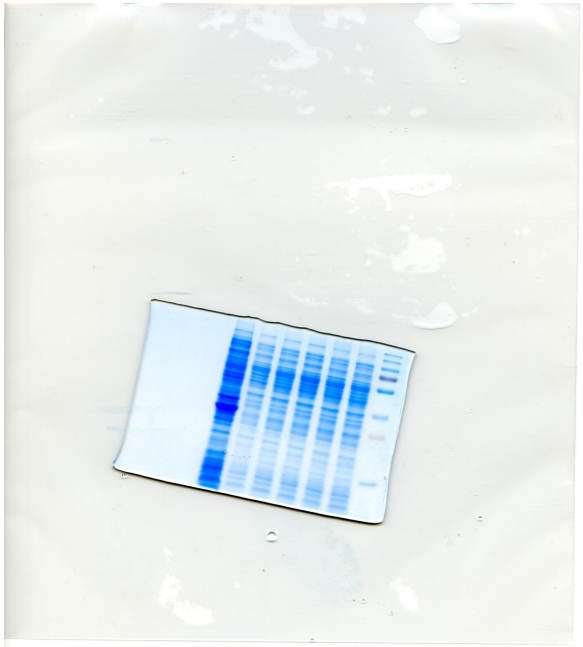
WEDNESDAY, 21/9

SDS PAGES: (Loading: Marker, 37°C IPTG 500µM, IPTG 500µM, IPTG 100µM, IPTG 25µM)

1709 LS NEB turbo001.jpg

No real differences between ctrl and induced samples visible

2009 LS NEB turbo + GABO002.jpg



No real differences between ctrl and induced samples visible

Membranes were dried to store them.

cultures of NEB Turbo + pJBEI-6410 + pBbA5k-EPL95 in 10ml LB+Kan+Amp did not grow

inoculation of NEB Turbo + pJBEI-6410+ pBbA5k-EPL95 in 20ml LB+Kan+Amp

Protein export

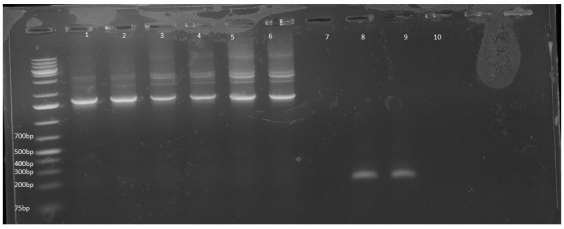
Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

WEDNESDAY, 21/9

New amplification of FliC due to a non-DNA-containing measurment of the frozen samples:
5µl MG 1655 colony (resuspended in 30µl H2O)
1µl 10mM dNTP
1µl FliC fw + FliC rev Gib (1:10)
1µl Phusion
10µl 5x Phusion Buffer HF
ad 50µl H2O

10' 98°C
30" 98°C |
30" 54°C |x30
1" 72°C |
5' 72°C

21.09.JPG



FliC can be found at lanes 8+9 with a length of 173bp. Marker: GeneRuler kb plus

Afterwards, performance of Gibson assembly of mRFP and FliC to create a template for amplification with corresponding primers for this fragment:
10µl Gibson MM
1µl purified FliC (185bp with 131 ng/µl - concentration 1.089pmol → diluted 1:4)
2µl purified mRFP (708bp with 56.3 ng/µl - concentration 0.122 pmol)
7µl H2O

For 20' on 50°C

INSERT PICTURE

Gel extraction and purification of the desired fragment was not succesful.

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

THURSDAY, 22/9

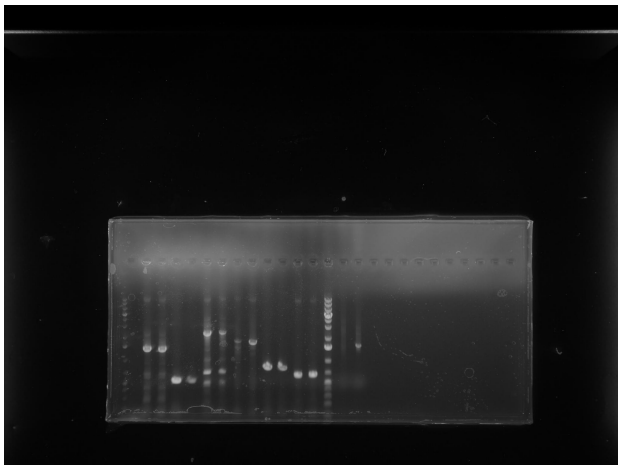
Cultures did not grow. Restreaked plate on new Kan + Amp plate and stored it at 37°C

Picked 2 more colonies of the transformation plate and inoculated those in 10 ml LB + Kan+Amp. Stored them at 37°C over night

Executed another pcr assembly of GcABC-G1 (second lane from right) and p450 (last lane). The conditions have not been changed.

5µl of the products were loaded into a 1% agarose gel.

0510 GcABC P450 .JPG



A fragment at 3000bp (Generuler 1kb plus) is visible for P450.

Pcr clean up of the P450 sample.

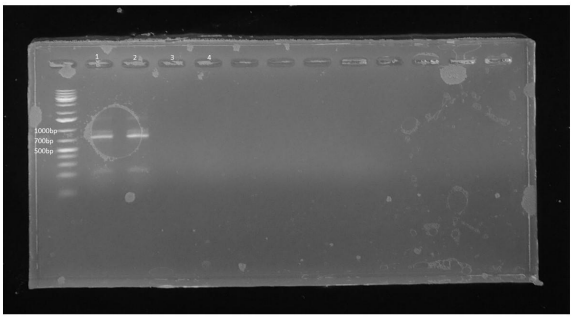
Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

THURSDAY, 22/9

Repitition of Gibsom assembly using the same conditions.

22.09.JPG



vague band above 700bp (=873bp) represents fused FliC-mRFP (below mRFP and FliC alone). Marker: GeneRuler kb plus

This time the gel extraction was succesful, purified fragment was stored at -20°C.

Production

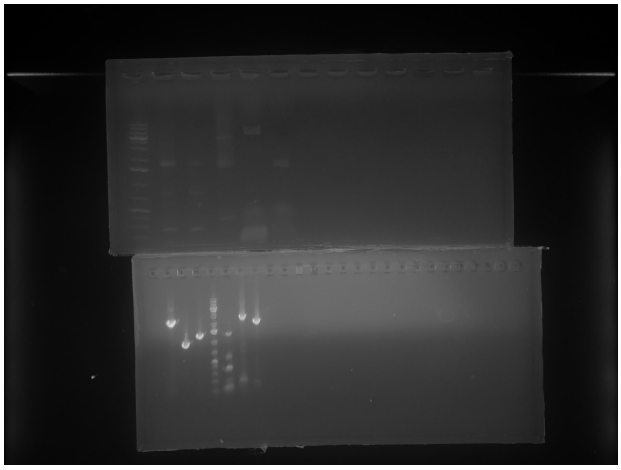
Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

FRIDAY, 23/9

New cultures did grow. Streaked both on new LB Kan+Amp plates and stored them on 37°C.

Prepared pcr with the assembled P450 (undiluted, lane1 upper gel; diluted to 1ng, lane2 upper gel) with primers with overhang for gibson assembly.

0610 P450insert P450dil insert GcABC.JPG



Slight bands around 3000bp (Generuler 1kb plus) visible in both lanes, but due to the low amount loaded into big wells (5µl) the intensity is very low.

PCR clean up (zymo) of both samples.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

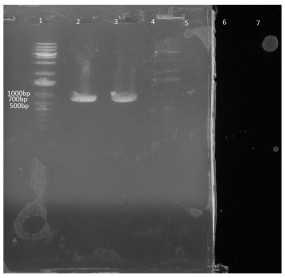
FRIDAY, 23/9

PCR of purified FliC-mRFP DNA using the corresponding primers:

1 resp. 3µl FliC-mRFP template
1µl 10mM dNTP
1µl FliC fw + mRFP rev (1:10)
1µl Phusion
10µl 5x Phusion Buffer HF
ad 50µl H2O

5' 98°C
30" 98°C |
30" 54°C |x33
1'20" 72°C |
5' 72°C

23.09.09.jpg



Fused fragment runs at ~850bp. Marker: GeneRuler kb plus

Succesfully amplified fragments were purified using the Zymo PCR cleanup kit.

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

SATURDAY, 24/9

Wrong digestion of PMFM027 backbone with PmeI (leads to integration cassette)
1h at 37°C
heat inactivation 20' at 65°C

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

SUNDAY, 25/9

Inoculation of new clone limonene production cultures 1 and 2 in LB Kan Amp 10ml

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

MONDAY, 26/9

yeast transformation into chemocompetent YMFM with P450 with overhangs.

plate 1:10000 dilution on Dropout plates + Leu and storage in 30°C

inoculation of 100ml EZ-rich medium with the Limonene cultures (4 flasks each)

induction of EZ-rich media cultures at OD 1,4 (#1) and 1,55 (#2) with different levels of IPTG (500, 100, 25 and 0 µM)
Overlay of 10ml dodecane

Inoculation of new clone limonene production cultures 3 and 4 in LB Kan Amp 10ml

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

TUESDAY, 27/9

Due to the unseccessful amplification of the mae1 synthesis (see 08/18), we amplified the gen from genomic S. pombe strains 2698(lov)872h- and 2699(lov)975h+ DNA.
Amplification of mae1 for Yeast expression plasmid using primers iGEM1 mae1 fwrd fwrd and iGEM mae1 rev
Amplification of mae1 for Operon plasmid using perimers mae1.2 fwrd and mae1.2_save_rev.

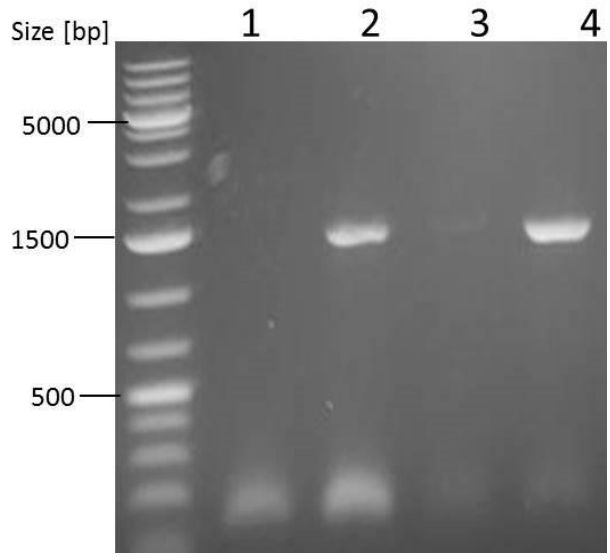
The genomic yeast DNA was extracted using the protocol from Marko Lõoke (2011) as follows:
1. Spin down 100-200 µl of liquid yeast culture (OD600=0.4). Suspend cells in 100 µl of 200mM LiOAc, 1 % SDS solution.
2. Incubate for 5 minutes at 70°C.
3. Add 300 µl of 96-100 % ethanol, vortex.
4. Spin down DNA and cell debris at 15 000 g for 3 minutes.
5. Wash pellet with 70 % ethanol
6. Dissolve pellet in 100 µl of H2O and spin down cell debris for 15 seconds at 15 000 g.
7. Use 1 µl of supernatant for PCR.

and the following PCR setup:

10' 98°C initial denaturation
30" 98°C denaturation |
30" 61°C annealing | x32
50" 72°C elongation |

Estimated band lenghts 1397 bp.

mae1_pcr.JPG



Positive bands for both mae1 for the expression plasmid and the operon plasmid.

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

TUESDAY, 27/9

harvesting the first ml of dodecane of the induced cultures and store the samples at 4°C

Inoculation of 4 flasks for each clone picked (Induction of those cultures #3 at OD600: 1 ; #4 at OD600: 0,7) in EZ rich medium overlay of 10ml of dodecane

LED screen of the fluorescing yeast transformation was negative. That means no introduction of the P450 gene into the LEU locus

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

WEDNESDAY, 28/9

Pcr of the backbone with wrong primers amplified the mturquoise cassette of the MFM027 backbone

Harvesting of the several dodecane layers (2nd for 1 and 2 and first for 3 and 4)

inoculation of blot membranes in anti his antibody (mouse) 1:10000in blocking buffer for one hour

washing the membranes with 1x TBST 3x 5min

inoculating the membranes in 2nd antibody (1:3000 in 5% milk) over night at RT

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

WEDNESDAY, 28/9

Inoculation of 4x 4ml culture tubes with BL21 pET28b YebF. Induction with different IPTG concentrations at oD 0.6:
10µM, 40µM, 100µM, 400µM

1ml samples were taken right before induction, 2h after induction and 18h after induction. The samples were centrifuged and 80µl supernatant transferred to another tube, pellet was resuspended in 80µl H2O. Each sample mixed with 20µl SDS-Buffer.

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

THURSDAY, 29/9

developing the membranes with ecl (1min) for 30 minutes.

blots were negative.

Production

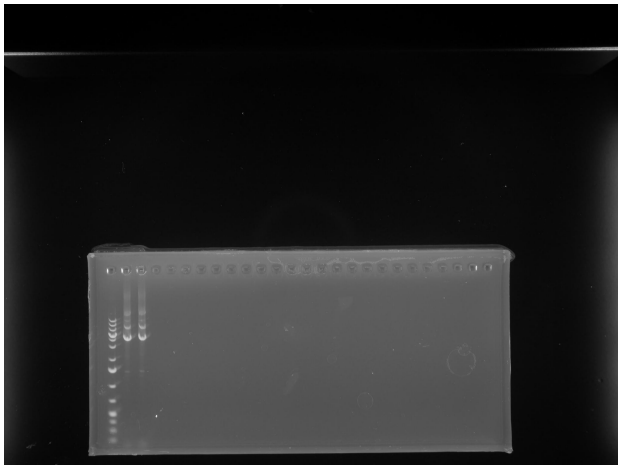
Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

FRIDAY, 30/9

stripping membrane in stripping solution (700µl b-mercaptoethanol, 10 ml pH6,5 50mM Tris-Hcl, 20ml 10%SDS up to 100ml with water)
30 minutes on 60°C
washing with TBST and blocking in milk

PCR of the backbone with the right primers:

P1000379.JPG



backbone visible around 8000kb (Generuler 1kb plus).

pcr cleanup

gibson with the P450 fragment and the backbone (30 minutes 50°C, inactivation afterwards)

transformation into NEB turbo plating on AMP plates

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

SATURDAY, 1/10

Efficiency test of spheroplast preperation (in all used media) and recovery in different media (1M Sorbitol in YPD, 1M Sorbitol without adjusted pH) at different timepoints through 1:100 dilution in H2O. Spheroplasts should undergo lysis where cells with cell wall should remain intact and therefore highten the oD.

Table22

	A	B	C	D	E	F	
1	Timepoint or Medium	RegMed	RegMed	RegMed	RegMed	Sorb	S
2	H2O	0,16					
3	Softening Medium	0,116		0,089			
4	Spheroblasting Med	0,122					
5	SpherMed + Zymolyase (10')	0,044	0,005	0	0,012		
6	SpherMed + Zymolyase (30')	0	0	0	0,01		
7	SpherMed + Zymolyase (60')	0,006	0,004	0,018	0,022		
8	120'	0,001	0	0	0	0,008	0
9	180'	0,005	0,013	0	0,005	0,024	0
10	220'			0,008	0,014	0,025	0
11	17h			0,015	0,014	0,017	0

Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

SATURDAY, 1/10

inoculation of 8 colonies into 15ml LB amp

miniprep of cultures that were spun down

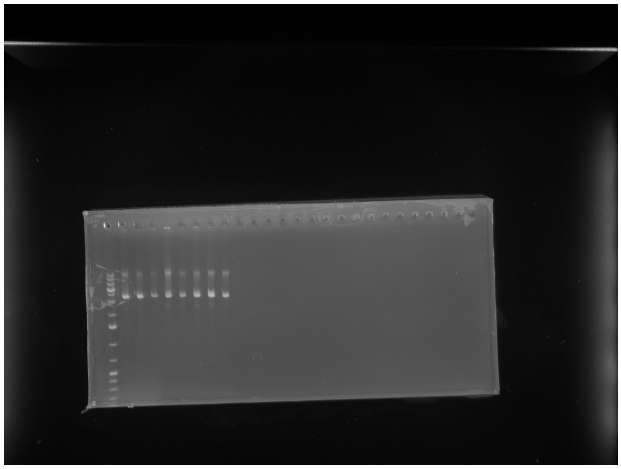
Production

Project: iGEM 2016
Authors: Steffen Lütke
Dates: 2016-07-25 to 2016-10-02

SUNDAY, 2/10

digestion of plasmids with PmeI (1h 37°C and inactivation 65°C 20')

P1000388.JPG



no positive band around 8000bp (Generuler 1kb plus). That means no cassette that could be transformed into yeast.

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

TUESDAY, 4/10

Gibson Assembly of construct Expression Plasmid and transformation in NEB Turbo on Amp plates.
Fragments:
iGEM1.1
iGEM1.3 (Backbone)
MatB
Mae1
No colonies the next day
Transformation will be repeated.

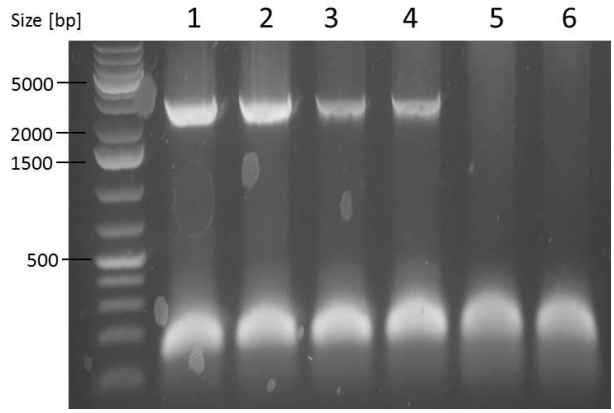
Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

WEDNESDAY, 5/10

Repeating the amplification of ppc gene from synthesized DNA product with new ordered primers.
The PCR protocol was similar as on 09/09.
To ensure the success of the amplification we used different anounts of tamplate (1, 3 and 5 µL) and every second PCR with additionaly 1 µL DMSO.
Estimated band lenghts of 2698 bp.

PCR_PpC.jpg



DMSO did not improve the yield of the PCR (lane two, four and six).
An increase of tamplate lowered the yield (lane one, three and five).
Gen band of lane one and two was cut out from the gel and cleaned using the Zymoclean™ Gel DNA Recovery Kit.

Gibson Assembly of construct Gibson2 and transformation in NEB Turbo on Amp plates.
Used fragments:
ppc_save
mae1_save
yne1
Backbone (pUC19)

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

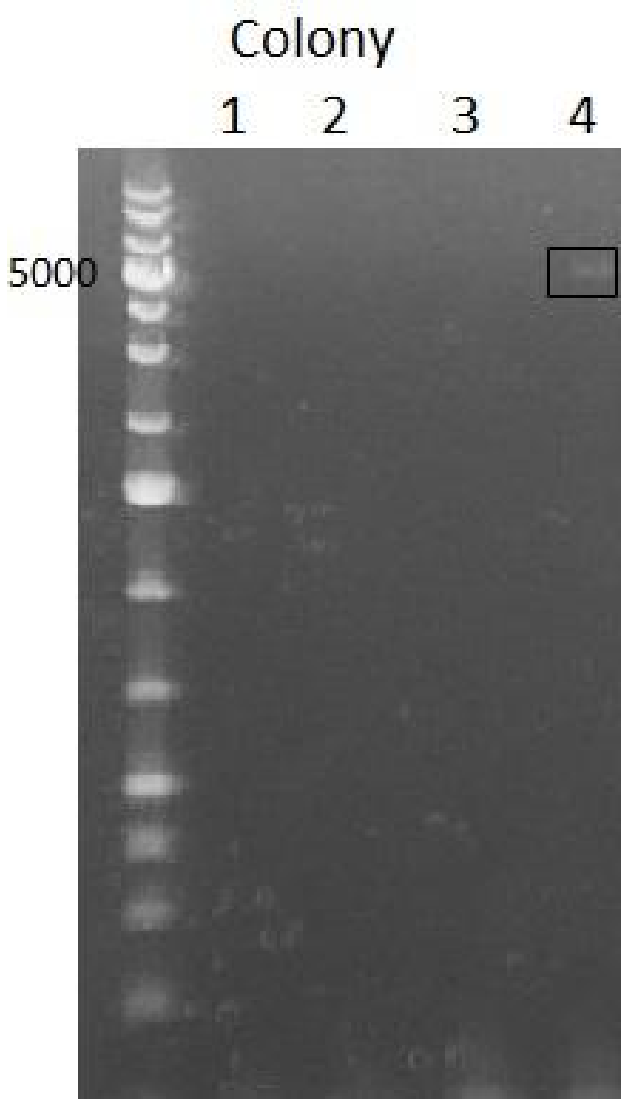
THURSDAY, 6/10

transformation was successful.
Four colonies were checked by colony PCR using following protocol:

5 µl template (1 colony diluted in 30 µL H2O)
2.5µl ppc fwrd primer
2.5µl mae1.2 rev primer
25 µL DreamTaq Green PCR Master Mix (2X)
15 µl H2O

and the following PCR setup:
10' 95°C initial denaturation
30" 95°C denaturation |
30" 58°C annealing | x32
2.5' 68°C elongation |
7' 68°C terminal extension

colony_PCR_Gibson2.jpg



Colony PCR of Gibson 2 transformants with ppc fwrd and mae1.2 rev estimated band at 5433bp.
There is a small band at the right lengths at colony 4. Continuing with this colony.
Miniprep of colony 4 to gain plamsid Gibson 2.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

THURSDAY, 6/10

Digestion and ligation of pASK_IBA7_NTAG_FNRCr and gibson assembled FliC-mRFP with XbaI and PstI in NEB Buffer 3.1.
1µl each enzyme
5µl buffer
1µg DNA (1.05µl for pASK_IBA7_NTAG_FNRCr and 20µl for FliC-mRFP)
ad 50µl H2O

Ligation set up as following:
1µl buffer
1µl T4 Ligase
1.7µl Vector
6.3µl Insert

Transformation into MG1655 with the usual protocol:
Unfreezing at 4°C
add 5µl Ligation
10' 4°C
1' 42°C
5' 4°C
add 200µl prewarmed LB
1h 37°C
afterwards, streak ~150µl on LB-Amp plate and let incubate over night.

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

FRIDAY, 7/10

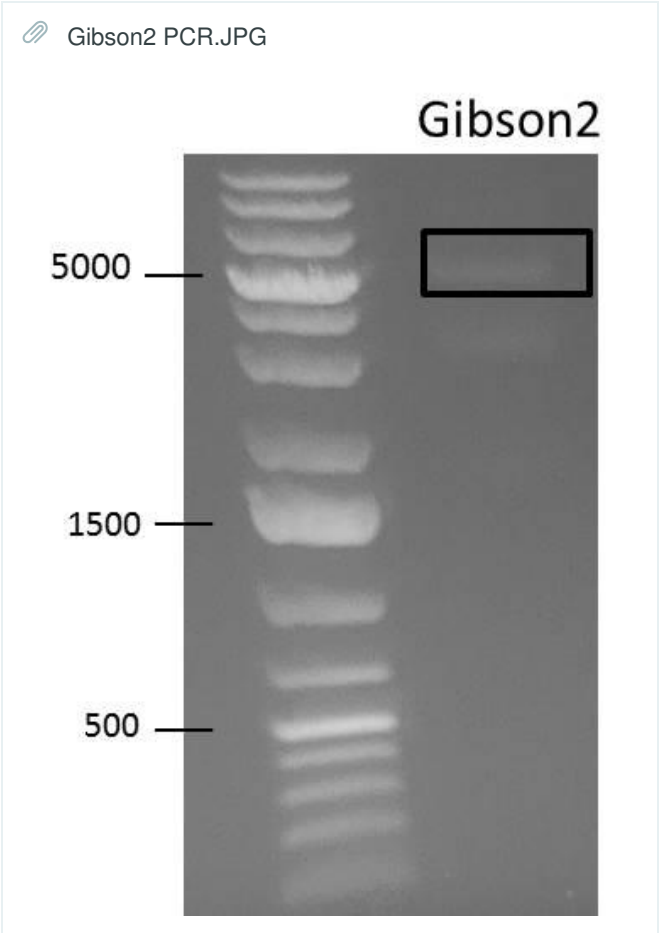
For the final Gibson assambly of the operon a genfragment from Gibson 2 was amplified by PCR including the gens ppc, mae1 and yne1:

1µl template (~ 1 ng)
- 2.5µl ppc fwrd primer
- 2.5µl mae1.2 rev primer
- 2µl dNTPs
- 10µl 10x phusion high fidelity buffer
- 31.5µl H2O
- 0.5µl Phusion polymerase.

and the following PCR setup:
- 1' 98°C initial denaturation
- 30" 98°C denaturation |
- 30" 61°C annealing | x32
- 2.5' 72°C elongation |
- 7' 72°C terminal extension

Estimated band lenghts 5421 bp.

Gibson2 PCR.JPG



The right band was cutted from gel and purified.

PCR iGEM1.3 was repeated with NK365 and NK366 as primers.
Estimated lengths 5990bp.

Gibson Assembly Expression plasmid and Gibson 3 (Operon) transformation into MG1655. (Gibson 3 Cam resistance, Expression Amp Resistance)

(Saturday) No Colonies on Expression Plate (Probably due to wrong antibiotica).
Fully grwon Cam Plates. (MG strain might be resistant against Chloramphenicol).

Yeast Assembly Expression plasmid in Sen Pk.
3µl each (iGEM1.1, iGEM1.3 (Backbone), MatB, Mae1) on SD-Trp plates.

(Monday) Many colonies on Yeast assembly plate.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

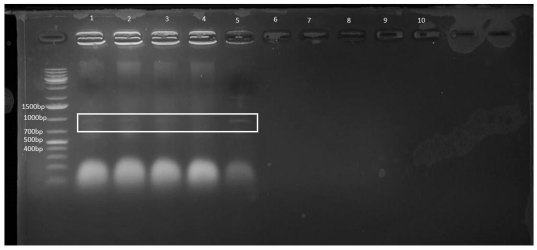
SATURDAY, 8/10

SDS gel of samples from 29.09. 20µl per sample were loaded, run for 50' on 180V/50mA.

---

5 Colonies of FliC ligation were picked and incubated in 5ml LB+Amp. To test whether the transformants contain desired insert, cPCR (before inoculation) and plasmid prep (M&N kit) were done.

cPCR pASK-FliCmRFP clones.JPG



Clones 1,2,3,5 showed expected bands at 860bp and were inoculated in 5ml LB +Amp. Marker: GeneRuler 1kb plus

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

SUNDAY, 9/10

Plasmids of clones 1-4 from FliC trafo were prepped (M&N kit). Concentration range of 40-68ng/µl.

Dependencies

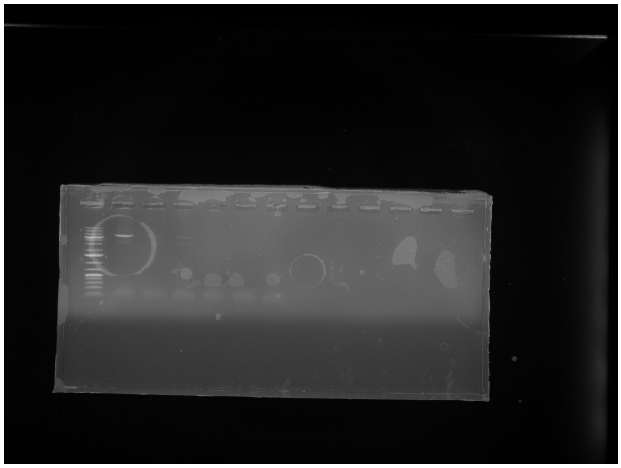
Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

MONDAY, 10/10

Inoculation 4 yeast colonies in sd-Trp media.
Transformation of Gibson assembly Gibson 3 (Operon) into NEB Turbo on CAM plates. (Hopefully this strain is not Chloramphenicol resistant).

PCR of Gibson1.3 (Backbon expression plasmid)
2 PCR settings (65°C and 69°C annealing temperature)
3 Samples each with 1µl, 3µl and 5µl Template.

PCR Gibson1.3 65 grad und 69 grad.JPG



PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

MONDAY, 10/10

Redoing PEG trafo protocol with following changes:

Spheroplasting conditions as already described with YMFM and S. pombe. Pombe showed no results measuring oD during the different steps (1:100 dilution in H2O).
After mixing yeast and bacteria (MG1655 pFAB or PCC 7002; both concentrated to oD of 20, so final oD is 10. This should be approx. 100x the cell number of yeast), followed by direct centrifugation (1000g, 5').
Next, 500µl 15% PEG were directly added to the pellet, resuspended, incubated for 15' on 30°C and centrifuged under the same conditions.
Instead of resuspending the pellet directly in regeneration medium, it was resuspended twice in different volumina of PEG. First in 250µl as first step and after centrifugation in 100µl (filled up to 1ml with corresponding regeneration medium after both steps).
The suspension was directly prepared for micoscropy.

Additionally, the survival of yeast spheroplasts in 1M Sorbitol pH 4.1 was tested.

Black spots within yeast seem to appear quite often, but not in the control. Could be dying coli. Or something else. Will investigate.
Regeneration medium with pH 4.1 seems to be sufficient for yeast (no differences to both normal pH controls) and will therefore be tested for the whole protocol.

Protein export

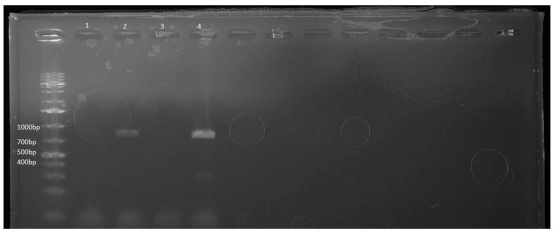
Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

MONDAY, 10/10

PCR with FliC fw + mRFP rev primers to verify insert.
1µl template plasmid (diluted to 1n/µl)
1µl fw+rev primer (1:10 dilution)
8µl H2O
10µl DreamTaq MM

5' 95°C
30" 95°C |
30" 56°C |x30
50" 68°C |
5' 68°C

pASK-FliCmRFP test PCR.JPG



Bands 2 + 4 (clones 2 and 5) showed best results with bands at expected 860bp. The other prepped plasmids were thrown away.
Marker: GeneRuler 1kb Plus

Western Blot of SDS page from 8.10.

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

TUESDAY, 11/10

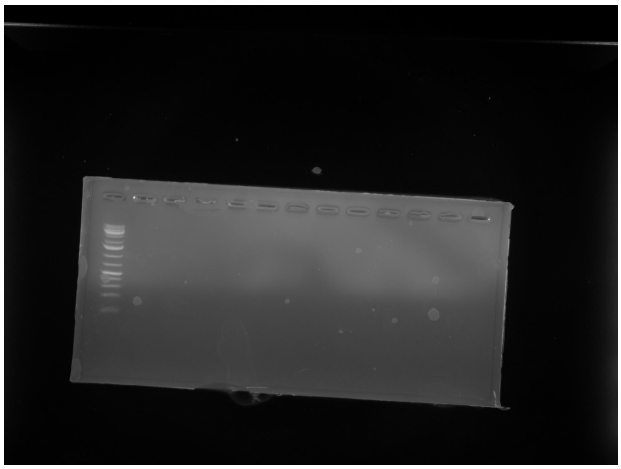
None of the Yeast? colonies grew in sd-trp media. (fail)

10 colonies on LB Cam plates from transformation of Gibson assemlby 3 (Operon).
6 Colonies were streaked on new plates and inocultated in 5 ml LB Cam for plasmid prep.

Clean up of amplified iGEM1.3 fragment and Gibson assembly of expression plasmid with new iGEM1.3 fragment.
Transformation in NEB Turbo on LB Amp plates.

PCR of iGEM1.1 since there is nothing left. (Nk367/368, 65°C, 1 min).

pcr igem1.1.JPG



It obviously did not work!!

PEG fusion

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-04-03 to 2016-10-11

TUESDAY, 11/10

Confocal micoscropy with samples from day before. Fixation was done on normal 1.5% agar pads, so possible positive events have to be inside of regenerated yeast cells (spheroplasts would undergo lysis under these conditions).
Six positive events could be detected with samples from different conditions (PEG 3350 with coli, PEG 6000 with coli, PEG 3350 with PCC 7002, but only in 1M sorbitol with YPD.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

TUESDAY, 11/10

PCR with FliC fw + FliD rev (50bp overhang for corresponding gene) primer amplifying KanR from pET28b.
Following settings were NOT succesful:
1µl Primer (FliC fw + FliD rev 1:10 dilution)
1µl pET28b (diluted to 1ng/µl)
1µl Phusion
1µl DMSO
1µl 10mM dNTP
10µl 5x Phusion buffer
ad 50µl dH2O
a)
5' 98°C
30" 98°C |
30" 54°C |x30
1'30" 72°C |
5' 72°C
b)
5' 98°C
30" 98°C |
30" 54°C |x15
1'30" 72°C |

30" 98°C |
30" 68°C |x15
1'30" 72°C |
5' 72°C
c)
5' 98°C
30" 98°C |
30" 54°C |x15
1'30" 72°C |

30" 98°C |
30" 68°C |x15
1'30" 72°C |
5' 72°C
d)
5' 98°C
30" 98°C |
1'20" 58°C |x33
1'30" 72°C |
5' 72°C
e)
5' 98°C
30" 98°C |
30" 56°C |x10
1'30" 72°C |

30" 98°C |

4' 72°C |

Dependencies

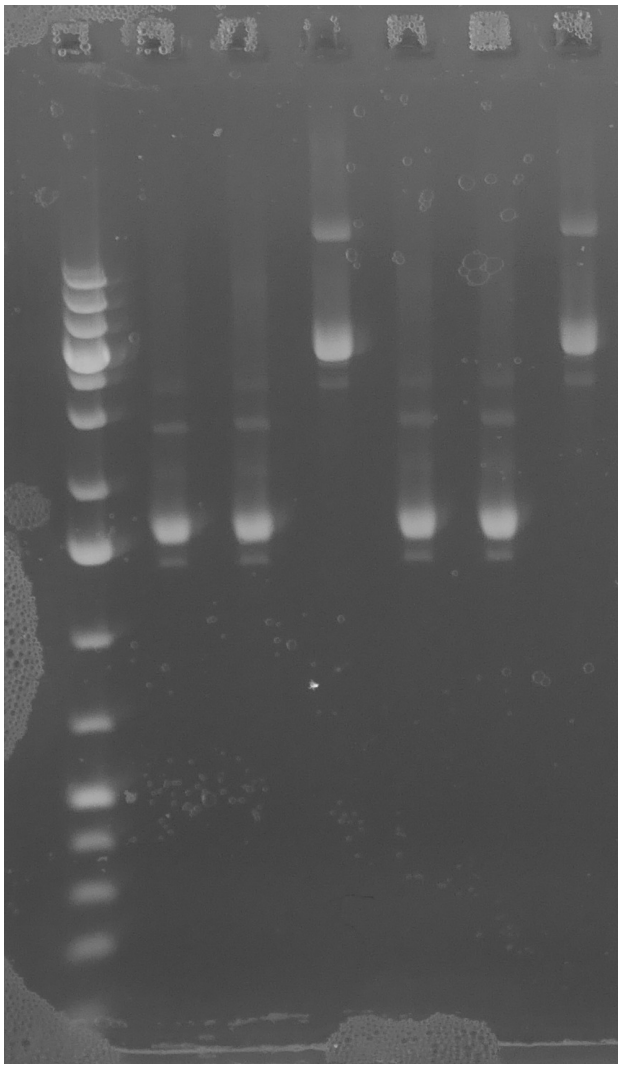
Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

WEDNESDAY, 12/10

Plasmid Miniprep of 6 Gibson 3 (Operon) transfomants.
Testrestriction with BamHI:
1µl DNA
1µl Enzyme
3µl 10x Cutsmart buffer
H2O up 30 µl total
on 37°C for 1 h

Estimated band lenghts:
194bp
837bp
1819bp
8089bp

testrestriktion BamHI Gibson 3 (operon).tif

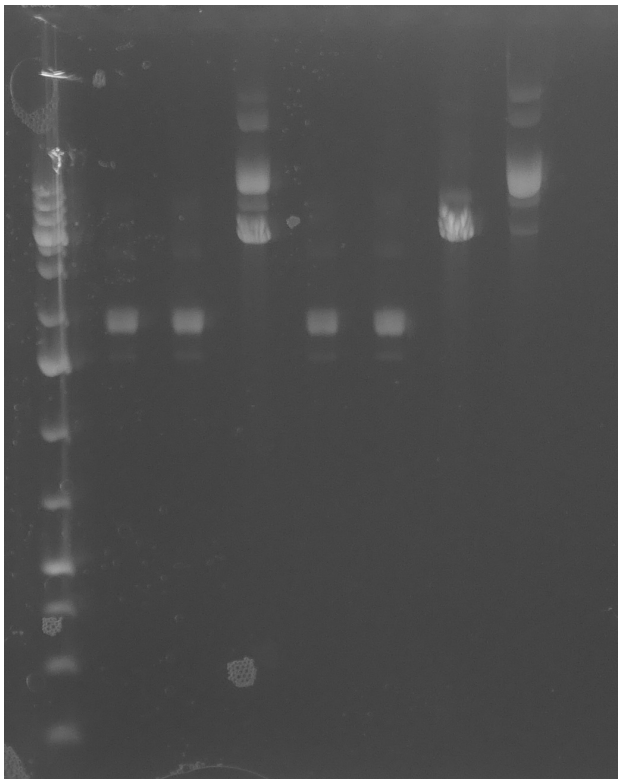


Testrestriction with XhoI and BglII:
1µl DNA

1µl each Enzyme
3µl 10x NEB 3.1 buffer
H2O up 30 µl total
on 37°C

estimated band lengths:
3003bp
7928bp

Testverdau operon xho1 bglii.tif



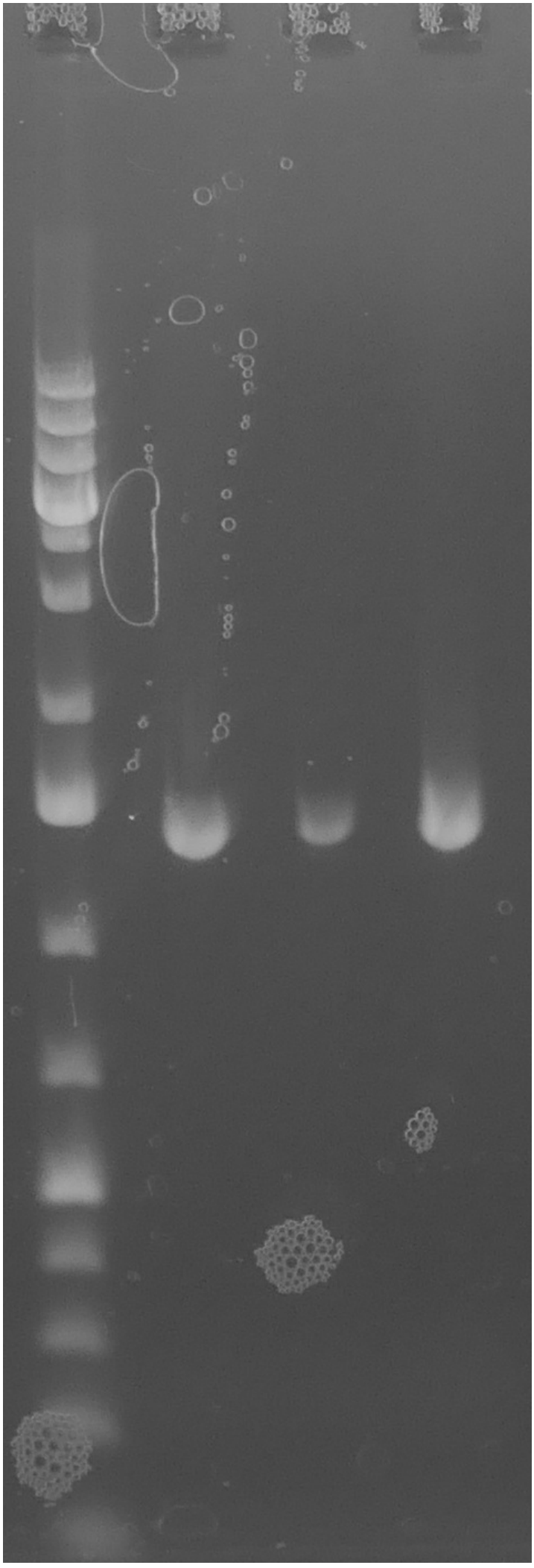
-Inoculating last 4 colonies from Gibson 3 (Operon) tranformation from Monday 10.10. in 5ml LB Cam for plasmid miniprep.

-There was only one colony on amp plate of the transfomants from the gibson assembly of the expression plasmid.
The colony were inoculated in 5 ml Lb Amp for Plasmid Miniprep.
Repeating transformation with Gibson assembly (Expression plasmid).

PCR iGEM1.1 (promoterregion)
1µl, 3µl, 5µl template
10µl phusion buffer 5x
2,5µl NK367
2,5µ NK368
2µl dNTPs
1µl DMSO
1µ Phusion

estimated band lenghts:
1407bp

PCR igem1.1 12.10.tif



worked!

Test PCR of colony 6 since it seems to be the most promising (operon).
Primer: Backbone fwrd/panD rev for an estimated band lenghts of 2352bp. Annealing Temperatur: 61.8°C, extension time: 1min 30s.
Primer: Backbone rev/ pa0132 fwrd for an estimated band lenghts of 8939bp. Annealing tmperature: 57°C, extension time: 7min

Protein export

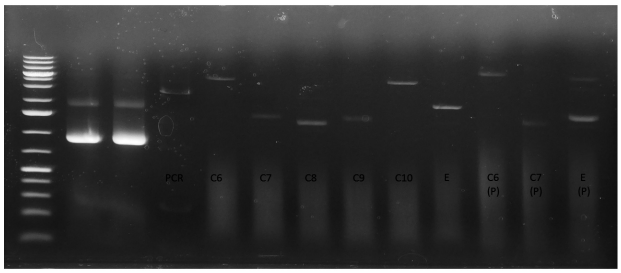
Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

WEDNESDAY, 12/10

Repitition of PCR.
1µl pET28b (diluted to 1ng/µl)
1µl Primer (FliC fw + FliD rev 1:10 dilution)
25µl Q5 2x MM (NEB)
ad 50µl dH2O

5' 98°C
30" 98°C |
40" 61°C |x30
1'10" 72°C |
5' 72°C

testpcr kleine fragment colony 6, test restriktion kolonie 7-10 mit xhoI und BglII, Expressionsplasmid verdau mit PvuI.tif



First two lanes after marker show succesful amplification with bands at expected 916bp. Marker: GeneRuler 1kb plus

---

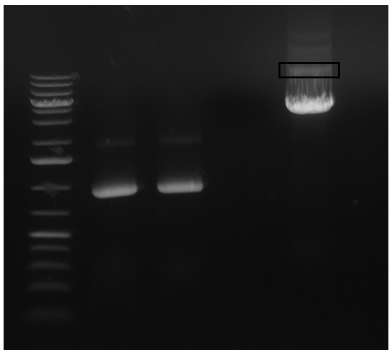
Inoculation of 15ml LB ON culture of E. coli AB330, which is capable of homologous recombination at 28°C

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

THURSDAY, 13/10

test pcr operon colony 6.tif



PCR of the short fragment (2352bp) did not work due to wrong cycler settings (my bad).
I told you so (Nikolai).
PCR of the long fragment shows a clear band at roughly 9kb which was estimated. The big band seems to be polution since it was seen at the restrictions as well.
For the short fragment we used the Taq polymerase of the GreenTaq Mastermix.
For the long fragment we used the NEB Q5 Polymerase for long amplifications.

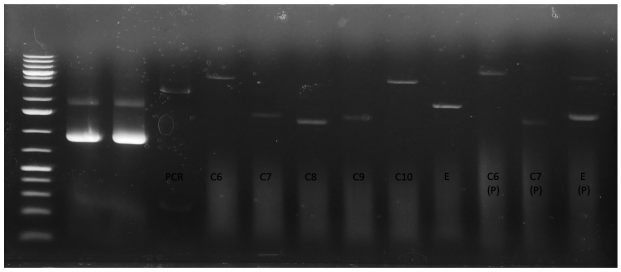
PCR for the short fragment (2352bp) will be repeated with correct cycler settings.

Plasmid Miniprep of 4 remaining colonies from the gibson 3 (Operon) transformation in NEB Turbo.

Testrestriktion of the 4 remaining colonies plus colony 6 with XhoI+BglII. Estimated band lengths: roughly 8000bp and 3000bp.

Tesrestriktion of expressionplasmid transformant with PvuI. Estimated fragment lenghts: roughly 6700bp and 3400bp.

testpcr kleine fragment colony 6, test restriktion kolonie 7-10 mit xhoI und BglII, Expressionsplasmid verdau mit PvuI.tif



Short fragment of colony 6 confirmed by PCR (lane one and two).
Plasmids seems to be unrestricted (last three lanes).
Continuing with colony 6 due to the righht fragments with the PCRs and with colony 10 since it looks similar.
Colonies 6 and 10 were inoculated for tomorrows procedure.

Two colonies from expressionplasmid transformation in NEB turbo from 12.10 were inoculated for plasmid miniprep
Yeast transformation with preped expression plasmid. Because why not (to safe time).
Following solution for the trafo was mixed and used:
260 µL PEG 3350
36 µL 1M LiAc
10 µL salmonsperm (prior to that heating for 10 min at 100°C)
1 µL (100 ng - 5 µg) DNA
52 µL H2O

Transformation protocol:

1. Thaw yeast cells at 4°C
2. 13000g 2 min and remove supernatant
3. Add 250 µL solution and resuspend
4. 42°C 40 min.
5. 13000g 30 sec. and remove supernatant
6. Resuspend in 1 mL H2O
7. Plate 200 µL on sd -trp

Test PCR of the Expression plasmid from transformation (10/11).
First PCR using the primers MatB clean fwrd/NK368 with dreamtaq master mix for an estimated band lenghts of 2905bp.
Second PCR using the primers NK365/MatB clean rev with Q5 Polymerase mix for an estimated band lenghts of 7477bp.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

THURSDAY, 13/10

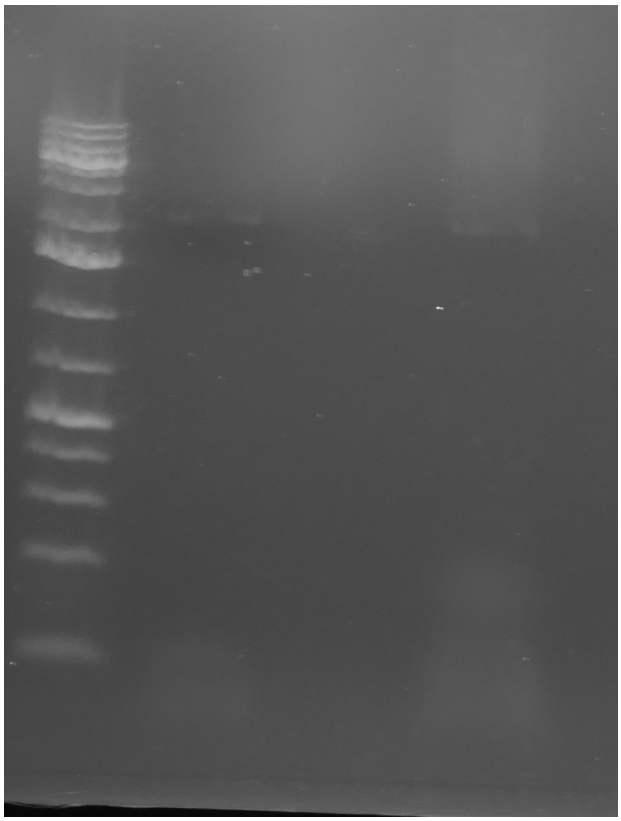
Inoculation of 100ml LB with AB330, growth to oD600=0.5
Heat shock at 42°C for 30'
Cooldown on ice for 30'
Centrifugation (4°C) for 10' at 4000g
Washing in 5ml ice cold dH2O
Centrifugation (4°C) for 10' at 4000g
Washing in 5ml ice cold dH2O
Centrifugation (4°C) for 10' at 4000g
Resuspending in 10% sterile glycerole (cold)
Electroporation of 300µl AB330 with 1µl FliDC-KanR (c=113ng/µl), setting Ecl (?)
Regeneration with 500µl LB on 28°C for 1.5h
Plated 300µl on LB-Kan plates
Incubation overnight on 30°C

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

FRIDAY, 14/10

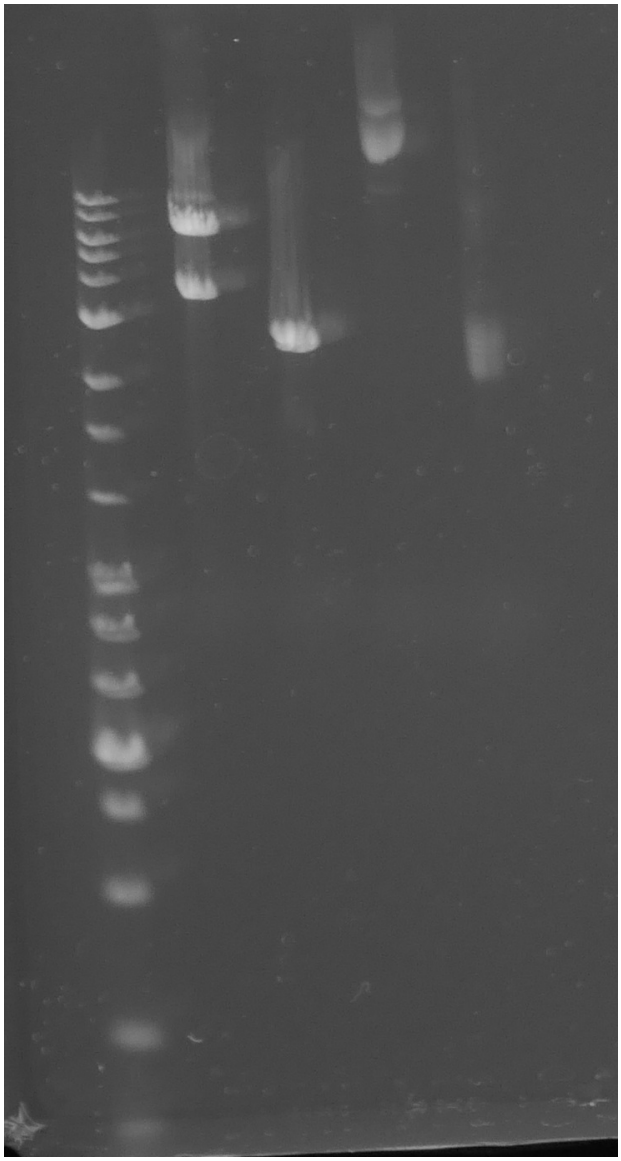
Test PCR Expression plasmid colony 1.tif



Both PCR samples showed bands at aproximately 2500bp - 3000bp, which means the first PCR outcomes is as expected; the second is not.

- testrestriction of remaining 2 expressionplasmid transformants (colony 2 and 3) with PvuI and an estimated band lenghts of 6700bp and 3400bp.

pvuI restriktion expressionplasmid kolonie 2 und 3 plus unverdaute plasmide.tif



Colony 2 seems to be correct (lane 1). We will proceed with this plasmid for yeast transformation.

Yeast transformation in YMFM with the expression plasmid of colony 2. Streaked on sd -trp plate (protocol from 10/13).

Preparing of HPLC samples:
Supernatant of overnight culture colony 6 and 10 of the operon plasmid transformants and the e. coli strain NEB Turbo as control.
Cell lysat of overnight culture of colony 6 and 10 of the operon plasmid transformants and the e. coli strain NEB turbo as control.

OD600:
C6: 3
C10: 2
Turbo: 2.4

12 ml culture were harvested and centrifugated at 5000g for 10 minutes.
1ml of the supernatant was transfered into 1.5ml eppi.
Cell pellet was resuspended in 5ml 10mM MOPS buffer.
Cells were lysated by sonification.
Cell suspension was spinned at 5000g for 10 minutes.
1ml of the supernatant was transferred into a 1.5 ml eppi.
100µl formic acid was added to the supernatant and the cell lysate.

samples were centrifuged at 17000g for 15 minutes.
50µl of the supernatant were used for UPLC-MS measurement.

The analyte was separated on a aminopropyl column (30 mm x 2 mm, particle size 3µm, 100 Å, Luna NH2, Phenomenex) using a mobile phase system comprised of 95:5 20 mM ammonium acetate pH 9.3 (adjusted with ammonium hydroxide to a final concentration of approximately 10 mM) / acetonitrile(A) and acetonitrile(B).
Chromatographic separation was carried out using the following gradient condition at a flow rate of 250 µl/min:
0 min 85% B; 3.5 min 0% B, 7 min 0% B; 7.5 min 85% B; 8 min 85% B.
Column oven and autosampler temperature were maintained at 15 °C.
The ESI source was set to the following parameters: Capillary voltage was set at 3.5 kV and nitrogen gas was used as nebulizing (20 psig), drying (13 l/min, 225 °C) and sheath gas (12 l/min, 400 °C). The QTOF mass detector was calibrated prior to measurement using an ESI-L Low Concentration Tuning Mix (Agilent) with residuals and corrected residuals less than 2 ppm and 1 ppm respectively. MS data were acquired with a scan range of 50-600 *m/z*.
Autorecalibration was carried out using 113 *m/z* as reference mass.
LC-MS data were analyzed using MassHunter Qualitative Analysis software (Agilent).

Samples:
C6 Supernatant - 1
C10 Supernatant - 2
Turbo Supernatant - 3
C6 Lysate - 4
C10 Lysate - 5
Turbo Lysate - 6

IGEM_1.CSV

IGEM_2.CSV

IGEM_3.CSV

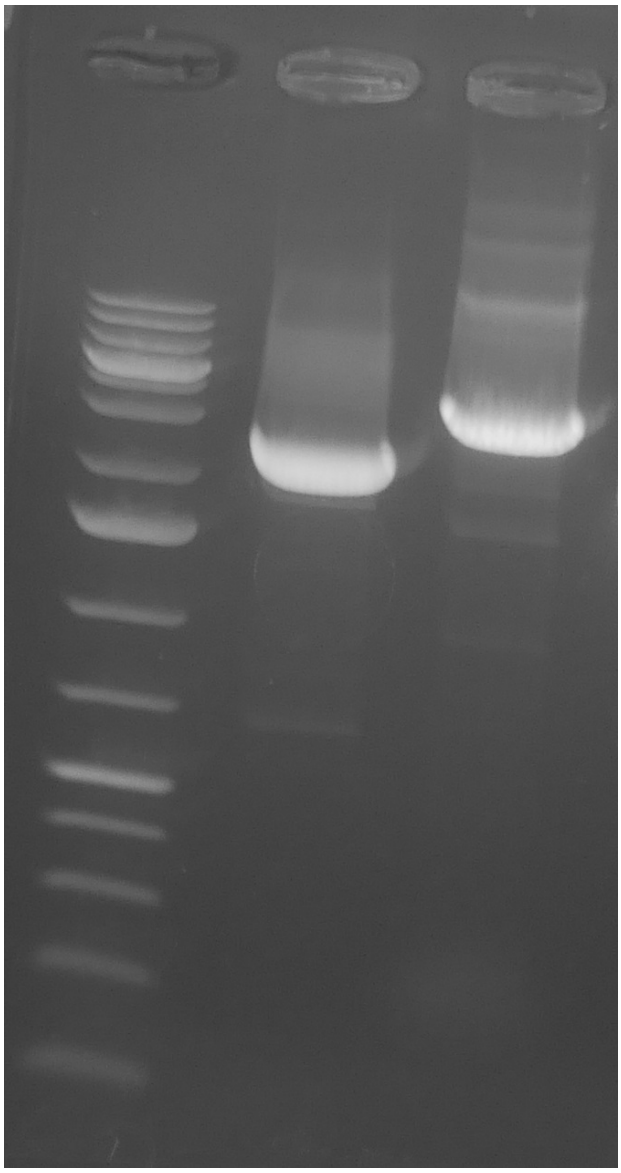
IGEM_4.CSV

IGEM_5.CSV

IGEM_6.CSV

Test PCR of colony 10 operon
with Backbone fwrd/PanD rev 61,8°C 1min 30s for an estimated band of 2352 bp
with Backbon rev/pa0132 fwrd 57°C 7min for an estimated band of 8939 bp.

test pcr operon colony 10.tif

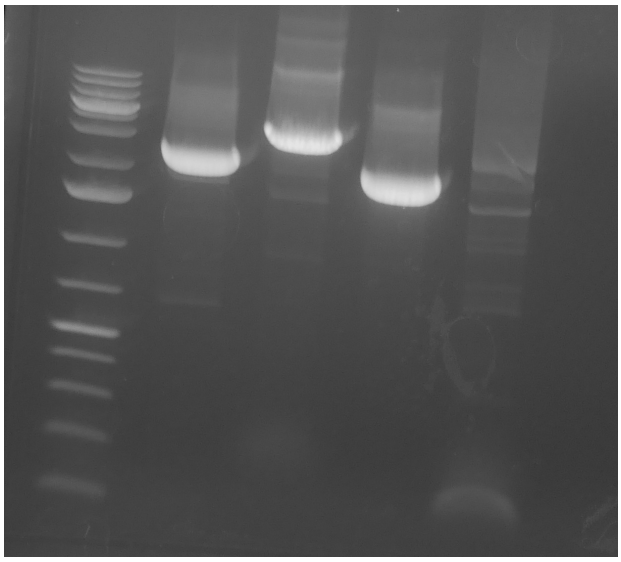


There seems to be correct 2352bp band but there is no 8939bp fragment.

Inoculating colony 6 and 10 operon in 15 ml LB plus cam for SDS Page.

Test PCR of the Expression plasmid of colony 2.
First PCR using the primers MatB clean fwrd/NK368 with dreamtaq master mix for an estimated band lenghts of 2905bp.
Second PCR using the primers NK365/MatB clean rev with Q5 Polymeraee mix for an estimated band lenghts of 7477bp.

Test PCR Expression plasmid colony 2tif.tif



There is the estimated band at 2905bp but no band at 7477bp.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

FRIDAY, 14/10

No clones could be detected

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

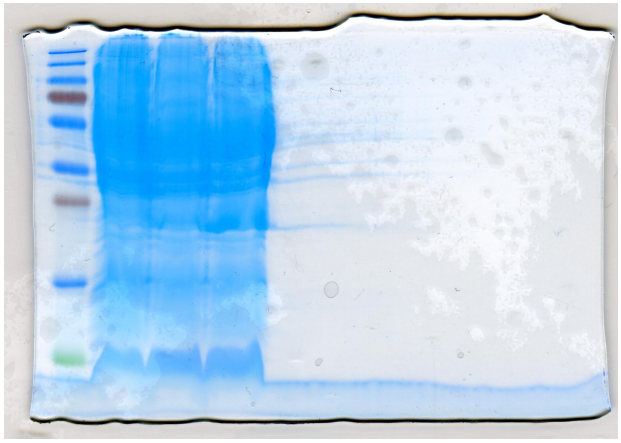
SATURDAY, 15/10

Preparing the samples (C6, C10, NEB Turbo) for SDS page.

Overnight culture of Operon colony 6 and 10 and NEB Turbo were harvested.
OD600:
C6: 3.18
C10: 2.4
Turbo: 3

12ml of the culture were centrifuged at 5000g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml H_2O.
75µl were transferred to a new eppi and 25µl 4x SDS buffer was added. The samples were heated at 98°C for 10 minutes and loaded on the gel.

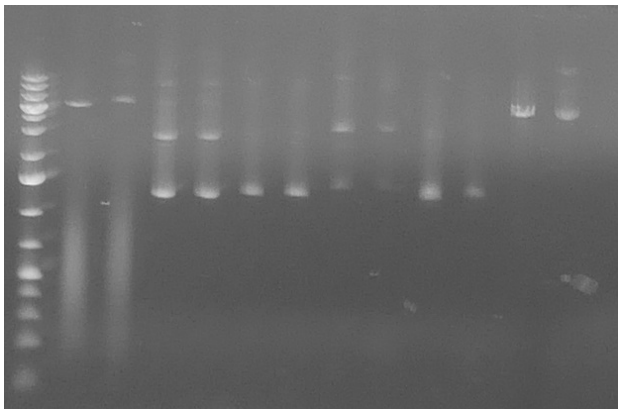
SDS page c10 c6 turbo.jpg



Unfortunate there is no difference between Turbo, C6 and C10. The used constitutive promoter might be not strong enough to see a difference in their proteomics.

Miniprep of additional operon transformants colony 10-15.
Testrestriction with XhoI and BglII
Estimated band lengths: roughly 8000bp and 3000bp.

Testrestriktion Xho1 BglII colonies 10-15.tif



Negative.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

SATURDAY, 15/10

Repitition of FliCD knockout using the same setting with an additional preperation of chemo competent cells (see standard protocol) after cool down on ice.
Different amounts of DNA were used with a range from 100 - 10 ng.

SDS page of YebF induction (clones from plate) with IPTG conc 100µM, 400µM, 800µM and 1mM.

PICTURES SDS PAGE

Dependencies

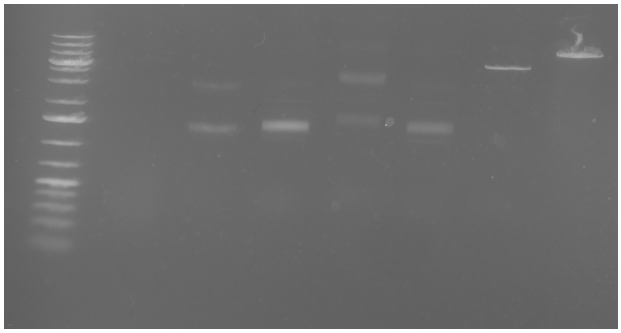
Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

SUNDAY, 16/10

Testrestriction of operon colonies 10-15 with EcoRI
Estimated band lenghts of 10932bp.

Testrestriction of expression plamsid colony 2 with XhoI
Estimated band lenghts of 10198bp.

testverdau operon c10-15 xhoI expression c2 ecori.tif



All operon bands were negative.
Expression band C2 seems to be positive (lane 7).

Transfomation of ymfm copetent cells with experession plasmid C2.
spin at 13000g for 2 min
resuspend in 350ml trafo mix (3335 PEG, Liac, Plasmid, ssDNA)
42°C for 40 min
spin at 13000g for 2 min
resuspend in 250µl h20
plate 200µl

- Inoculate operon colony 6, 10 and 15 for hplc and sds gel analyses.
2 times 15ml for each.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

SUNDAY, 16/10

Still no clones could be detected. Found out that the used setting at the gene pulser (Ec1) is 1.8kV instead of 2.5kV (Ec2). Repitition of the experiment with the right voltage and DNA amounts of 1000ng, 500ng, 100ng and 50ng.

Dependencies

Project: iGEM 2016
Authors: Nikolai Huwa
Dates: 2016-06-01 to 2016-10-17

MONDAY, 17/10

Preparing of HPLC samples.

Supernatant of overnight culture of Colony 6, 10 and 15 of the operon transformants and the e. coli strain NEB Turbo as control.

Cell lysat of overnight culture of colony 6 and 10 of the operon transformants.

OD600:
C6: 2.76
C10: 4.87
C15: 4.68

12 ml culture were harvested and centrifugated at 5000g for 10 minutes.
1 ml of the supernatant was transfered into 1.5 ml eppi.
Cell pellet was resuspended in 5 ml 10 mM MOPS buffer.
Cells were lysated by sonification.
Cell suspension was spinned at 5000g for 10 minutes.
1 ml of the supernatant was transferred into a 1.5 ml eppi.
100 µl formic acid was added to the supernatant and the cell lysate.
Samples were centrifuged at 17000g for 15 minutes.
50 µl of the supernatant were used for HPLC measurement.

The UPLC-MS method was the same as on 10/14.
Samples:
C6S - 1
C6L - 2
C10S - 3
C10L - 4
C15S - 5
C15L - 6

IGEM_161017_1.CSV

IGEM_161017_2.CSV

IGEM_161017_3.CSV

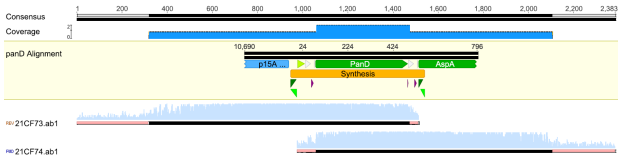
IGEM_161017_4.CSV

IGEM_161017_5.CSV

IGEM_161017_6.CSV

Preparing sequencing samples of Operon plasmid from colonies 6, 10 and 15 using primers panD fwrd and panD rev to demonstrate for succesful integration of panD into the transformed operon plasmid.
Sequencing samples were mixed after GATC recommendation.

panD sequencing.tif



Sequencing alignment shows that integration of panD in operon c6 was positive.

Preparing sequencing samples of Expresssion plasmid colony 2using primer matB_clean_fwrd and matB_clean_rev to sequence for succesful integration of matB into the assembled expression plasmid.
Sequencing samples were mixed after GATC recommendation.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

MONDAY, 17/10

No clones.
Already prepared pASK_IBA7_NTAG_FNRCr with inserted FliC-mRFP6xhis was transformed into BL21.

Thaw at 4°C
add 100ng Ligation
10' 4°C
1' 42°C
5' 4°C
add 200µl prewarmed LB
1h 37°C
afterwards, streak ~150µl on LB-Amp plate and let incubate over night.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

TUESDAY, 18/10

Colonies for pASK FliC-mRFP construct could be detected and were inoculated in 200ml LB-Amp. After growth to an oD of 0.7, cells were induced using tet on different concentrations. Samples were taken 2h and 4h after induction and put on put on SDS-page for Western blotting.

Surprisingly colonies for the fliCD knockout in AB330 were detected. These were picked and incubated in LB-Kan at 28°C. Since verification with PCR is not possible due to primer length, indirect verification by seeing adhesive cell aggregates due to the lack of flagella had to be sufficient.
Growth of the colonies took longer than expected, hence no transformation with the expression construct could be done before wiki freeze. This will be done the next days.

Protein export

Project: iGEM 2016
Authors: Patrick Gerlinger
Dates: 2016-05-20 to 2016-10-19

WEDNESDAY, 19/10

Western blotting showed neither expression nor export into extracellular space.